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Development of Toxicity Benchmarks and Bioaccumulation Data for N-based Organic Explosives for Terrestrial Plants and Soil Invertebrates

SERDP Project ER-1416

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National Research Council of Canada

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The main goal of this project was to develop toxicity benchmarks acceptable for Ecological Risk Assessment (ERA) of soil contaminated with 2,4-dinitrotoluene (2,4-DNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and nitroglycerin (NG), and for derivation of Draft Ecological Soil Screening Level (Eco-SSL) values for ecologically relevant soil biota.

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ADNT : Aminodinitrotoluene
2A-4NT : 2-amino-4-nitrotoluene
4A-2NT : 4-amino-2-nitrotoluene
ANCOVA : Analysis of covariance
ANFO : Ammonium nitrate-fuel oil
ANOVA : Analysis of variance
AP : Acid phosphatase
APG : Aberdeen Proving Ground
ASTM : American Society for Testing and Materials

BA-SSC : Bilogical activity-based soil screening concentration
BAF : Bioaccumulation factor
BAF_K : Kinetic bioaccumulation factor
BCF : Bioconcentration factor
BDL : Below analytical detection limit
BERA : Baseline Ecological Risk Assessment
BR : Basal respiration
BRI : Biotechnology Research Institute

CAS : Chemical abstract service
CI : Confidence interval
CL : Confidence limit
CL-20 : 2,4,6,8,10,12-hexanitro-2,4,6,8,10,12-hexaazaisowurtzitane
COPC : Contaminants of Potential Concern

DH : Dehydrogenase
DNG : Dinitroglycerin
DNT : Dinitrotoluene
DoD : Department of Defense

EBM : Earthworm bioaccumulation microcosm
EC : Environment Canada
ECBC : Edgewood Chemical Biological Center
Eco-SSL : Ecological Soil Screening Level
EC_{20} : Effect concentration that causes 20 percent inhibition
EC_{50} : Effect concentration that causes 50 percent inhibition
EM : Energetic materials
ERA : Ecological risk assessment

FLSD : Fisher’s least significant difference

HMX : Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
HPLC : High pressure liquid chromatography

INT : 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazoliumchloride

KCL : Kirkland clay loam
KL : Kirkland loam

LOAEC : Lowest observed adverse effect concentration
LOEC : Lowest observed effect concentration

MUB : Methylumbelliferyl

NA : Not available
NAG : N-acetly-glucosaminidase
ND : Not detected
NG : Nitroglycerin
NOAEC : No observed adverse effect concentration
NOEC : No observed effect concentration

OECD : Organisation for Eonomic Cooperation and Development

PAM : Plant accumulation microcosm
PAR : Photosynthetically active radiation
PN : Potential nitrification

R^2 : Coefficient of determination
RDX : Hexahydro-1,3,5-trinitro-1,3,5-triazine

SAS : Standard artificial soil
SD : Standard deviation
SERDP : Strategic Environmental Research and Development Program
SIR : Substrate-induced respiration
SLERA : Screening level ecological risk assessment
SSL : Sassafras sandy loam

THF : Tetrahydrofuran
TNB : Trinitrobenzene
TNT : Trinitrotoluene
TSL : Teller sandy loam

USEPA : United States Environmental Protection Agency
USGAO : United States General Accounting Office
USGS : United States Geological Survey

WCL : Webster clay loam
WHC : Water holding capacity

Abstract

Objectives: The main goal of this project was to develop toxicity benchmarks acceptable for Ecological Risk Assessment (ERA) of soil contaminated with 2,4-dinitrotoluene (2,4-DNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and nitroglycerin (NG), and for derivation of Draft Ecological Soil Screening Level (Eco-SSL) values for ecologically relevant soil biota. This main goal was achieved by addressing the following technical objectives:

1) Determining the toxicities of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG to soil invertebrates and terrestrial plants in soil with high bioavailability characteristics;
2) Examining the effect of various soil physical/chemical characteristics (*e.g.*, organic matter content, pH, clay content) on 2,4-DNT toxicity to terrestrial plants and soil invertebrates;
3) Determining the bioaccumulation potentials of 2,4-DNT, HMX, NG, TNT, and RDX from soil to invertebrates and terrestrial plants, respectively;
4) Determining data for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on the soil microbial activity endpoints and contrasting these data with the toxicity benchmarks developed using standardized single-species toxicity assays;
5) Developing Draft Eco-SSLs for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG for terrestrial plants and soil invertebrates, based upon concentration-response relationships.

Technical Approach: Studies were designed to develop scientifically-defensible toxicity data for the derivation of plant-based and soil invertebrate-based Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG, weathered-and-aged individually in soil. Ecotoxicological testing was specifically designed to meet the USEPA criteria for Eco-SSL derivation. Toxicity testing was also conducted with additional natural soil types to extend the range of soil physico-chemical characteristics that were hypothesized to affect the 2,4-DNT toxicity to soil organisms in order to investigate and characterize predominant soil physico-chemical parameters that can affect the bioavailability and resulting toxicity of 2,4-DNT to terrestrial plants and soil invertebrates. Assessment of soil microbial activity was included in the project to establish data on 2,4-DNT, 2-ADNT, 4-ADNT, and NG effects on critical ecosystem-level processes such as energy and nutrient cycling. Multiple soil microbial activity endpoints, including basal respiration, substrate-induced respiration, microbial biomass carbon, litter decomposition (orchard grass), and enzyme activities, were assessed to develop toxicity data for the derivation of Biological Activity-based Soil Screening Concentrations (BA-SSC) utilizing an approach similar to Eco-SSL derivation. Bioaccumulation studies were conducted to test the hypotheses that selected explosives released in soil can accumulate in soil invertebrates or terrestrial plants. Bioaccumulation factors (BAF) were determined from the ratio of the uptake and the elimination kinetic rate constants estimated during the uptake and elimination phases of a bioaccumulation test.

Results: Definitive studies using three plant and three soil invertebrate test species exposed in sandy loam soils established new ecotoxicological data for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG under conditions of very high relative bioavailability for organic chemicals in soil. These data were used to derive Draft Eco-SSL concentrations for each energetic material (EM). Soil-related differences were evident in both phytotoxicity benchmarks, and soil invertebrate toxicity benchmarks established in studies with 2,4-DNT. These studies identified soil organic matter and clay as the dominant properties mitigating 2,4-DNT toxicity for soil annelids (earthworms and potworms), and organic matter as the soil constituent mitigating 2,4-DNT toxicity for plants. Strong correlations were also detected for several annelid toxicity endpoints

and soil cation exchange capacity. The present studies showed that soil contamination with 2,4-DNT, 2-ADNT, 4-ADNT, and NG can alter the rates of biologically-mediated processes in soil by either inhibiting or stimulating the soil microbial activity at the affected sites. Basal respiration and dehydrogenase activity assays were the most robust among the soil functional tests used in the present studies, and allowed us to establish toxicity data for each of the four EM investigated in this project. Toxicity benchmarks determined in this project for EM effects on soil microbial activity endpoints were used for derivation of BA-SSC values. A summary of the draft Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, NG, and HMX determined from the growth toxicity benchmarks for terrestrial plants (alfalfa, barnyard grass, and ryegrass), and from reproduction toxicity benchmarks for soil invertebrates (earthworm, potworm, and collembola) is presented in the table below. The BA-SSC values for the four EM are also included in this table.

Energetic Material	Draft Eco-SSL for terrestrial plants (mg/kg)	Draft Eco-SSL for soil invertebrates (mg/kg)	Biological Activity BA-SSC (mg/kg)
2,4-DNT	6	18	104
2-ADNT	14	43	208
4-ADNT	33	18	84
NG	21	13	98
HMX	Not phytotoxic up to 10,000 mg/kg	16	Not determined

This project investigated bioaccumulation (earthworms) and bioconcentration (plants) potentials for TNT, 2,4-DNT, HMX, RDX, and NG in order to determine the respective factor values (BAF and BCF) to produce data that can be used to assess the potential risks to higher trophic levels through food web transfer of energetic soil contaminants. The BAF and BCF values shown in the table below were developed for each EM over a range of non-toxic exposure concentrations.

Energetic Material	BAF in earthworms	BCF in ryegrass shoots	BCF in ryegrass roots
RDX	7.35	175	75
HMX	No accumulation	14	1.1
TNT	No accumulation	No accumulation	No accumulation
2,4-DNT	No accumulation	1.8	0.7
NG	No accumulation	No accumulation	No accumulation

Benefits: Upon acceptance by the USEPA, the draft Eco-SSL values will allow screening of site-soil data to identify those contaminant EMs that are not of potential ecological concern and do not need to be considered in the Baseline Ecological Risk Assessment (BERA), resulting in significant cost-savings during site assessments. Benchmark data and draft Eco-SSL values developed in these studies have been transitioned to the USEPA, and reported to DoD constituensies. The Eco-SSLs, BA-SSCs, BAFs, and BCFs developed in this project will provide indispensable tools for installation managers to gauge the ecotoxicological impacts of military operations that involve the use of explosives, thus ultimately promoting the sustainable use of testing and training ranges.

1. Objectives of the project

The first goal of this project was to generate toxicity benchmark values for terrestrial plants and soil invertebrates that can be used for developing Draft Ecological Soil Screening Level (Eco-SSL) values for 2,4-dinitrotoluene (2,4-DNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-amino-2,6-dinitrotoluene (4-ADNT), octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX), and nitroglycerin (NG). Ecotoxicological testing was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Guideline (USEPA, 2005). This project aimed at developing draft Eco-SSL values using data generated from laboratory toxicity tests with test species that are representative of naturally occurring relevant ecological receptors. Receptor responses were coupled with appropriate measures of chemical exposure data to ensure that these draft Eco-SSLs are appropriately effects-based.

The second goal of this project was to determine the bioaccumulation or bioconcentration factors for TNT, 2,4-DNT, HMX, RDX, and NG using terrestrial plant and soil invertebrate species to produce data that can be used to assess the potential risks to higher trophic levels through food web transfer of energetic soil contaminants. We aimed at developing bioaccumulation factors for energetic materials (EM) from the soil into plants and earthworms over a range of non-toxic exposure concentrations. These data will provide input for risk assessors and site managers examining potential environmental impacts of EM in soil for avian and mammalian receptors.

In summary, the goals of this research were achieved by addressing the following technical objectives:

(1) Determine the toxicities of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG to plants and soil invertebrates in soils with high relative bioavailability characteristics;

(2) Examine the effect of soils with various physical/chemical characteristics (*e.g.*, organic matter and clay content) on EM toxicity to plants and soil invertebrates;

(3) Determine the bioconcentration and bioaccumulation potentials for 2,4-DNT, HMX, NG, TNT, and RDX from soil to plants and invertebrates, respectively;

(4) Determine data for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on the soil microbial activity endpoints and contrast these data with the toxicity benchmarks developed using standardized single-species toxicity assays; and

(5) Develop draft Eco-SSLs for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG for plants and soil invertebrates, based upon concentration-response relationships.

2. Project background

Energetic materials (EMs) are employed in a wide range of commercial and military activities, and frequently are released into the environment. The fate of these compounds is dependent upon the sum of all processes leading to their sequestration or elimination. Such processes include the rate of introduction, physical transport, chemical or biological transformations, and remediation efforts. For example, ammonium nitrate-fuel oil (ANFO) mixtures are widely used as explosives by the mining and construction industries, but the components of these products are rapidly metabolized by most microbial communities (USGS, 2002). Explosives TNT, RDX, and HMX are less biodegradable, and manufacture, use, or disposal practices have led to their persistence in the environment (Spain, 2000). Specific activities resulting in the significant release of EMs include: dumping of aqueous washings during manufacture (USEPA, 2003a), the failure of munitions to detonate on training ranges (Thiboutot *et al.,* 1998), deposition of ordnance in landfills (USEPA, 2003b), and the incomplete combustion of ordnance during disposal by open burning or detonation (USEPA, 2003c). These compounds have been identified in plants and earthworms exposed to contaminated soil (Groom *et al.*, 2002; Robidoux *et al.*, 2004b; Sunahara *et al*., 2009). Army ammunition plants were identified as the most heavily contaminated sites (USGS, 2002).

More than 15 million acres in the US are suspected or known to have been contaminated with elevated levels of EMs in soil (USGAO, 2003). As of 2002, the US Department of Defense (DoD) identified 2,307 sites of potentially contaminated by EMs. As of 2003, assessments were completed for only 558 of these sites, of which 83 required remediation with estimated costs ranging from $8 billion to $35 billion (USGAO, 2003). The DoD continues to identify additional sites associated with military operations. The effects of several of these EMs on soil biota have not been sufficiently investigated. This presents a challenge for Site Managers who wish to distinguish those sites that do not pose significant environmental risks from those that do, prioritize contaminated sites by the level of risk posed, quantify the risks at each site, and decide whether further investigation in a BERA is merited in order to determine appropriate remedial actions. Therefore, development of ecotoxicological benchmarks for EMs in soil has become a critical need in the US.

Ecotoxicological testing for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Guideline (USEPA, 2005). The Eco-SSLs are concentrations of contaminants in soil that are protective of ecological receptors that commonly come into contact with soil or ingest biota that live in or on such soils. These values can be used in the Screening Level Ecological Risk Assessment (SLERA) to identify those contaminants that are not of potential ecological concern in soils, thus do not require further evaluation in the BERA, potentially resulting in cost-savings during ecologically-based site assessments and remedial investigations.

2.1. Toxicity of energetics to terrestrial plants

Published studies on phytotoxicity of explosives to higher terrestrial plants are scant (Gong *et al.*, 1999b; Sunahara *et al.,* 2001; Hannink *et al.,* 2002; Robidoux *et al.,* 2003; reviewed by Sunahara *et al.,* 2009). Winfield *et al.* (1999) found that exposure to RDX (up to 4000 mg/kg soil) during

early life stage resulted in adverse responses in sensitive terrestrial plants such as sunflower and sainfoin. In a field study, corn, tomato, and lettuce died when exposed to 580 mg RDX/kg soil and 1720 mg TNT/kg soil (Price *et al.,* 1997; Pennington and Brannon, 2002). Wild-type tobacco plants exposed to 1 mM nitroglycerin could not germinate normally, and showed severe stunting of root and shoot development (French *et al.,* 1999). Although a screening benchmark of 100 mg RDX/kg soil was determined by Talmage *et al.* (1999), confidence in the benchmark is low because the available data are insufficient to establish an Eco-SSL.

The largest contribution to the knowledge of the toxicity of N-based organic EMs to plants came from research, including SERDP-funded projects, conducted by members of our combined team (Simini *et al.,* 1992; Rocheleau *et al.,* 1999, 2003, 2006; Renoux *et al.,* 2001; Sunahara *et al.,* 2001; Gong *et al.,* 2003; Kuperman, 2003; Lachance *et al.,* 2003). It has been demonstrated that RDX at concentrations of 100 mg/kg or more in soil caused significant biomass reduction in cucumber seedlings (Simini *et al.,* 1992). Results from the SERDP CU-1221 project (Rocheleau *et al.,* 2003) showed that RDX and HMX were not toxic at concentrations of 10,000 mg/kg using Sassafras sandy loam (SSL) soil. TNB, 2,4-DNT and 2,6-DNT reduced growth of alfalfa, barnyard grass, and ryegrass in freshly amended SSL soils and toxicity generally increased in weathered-and-aged EM amended soils. This knowledge will be applied to ecotoxicity testing as part of the proposed investigations.

2.2. Toxicity of energetic materials to soil invertebrates

Scant literature exists on the toxicity of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG to soil invertebrates, and discrepancies are often found regarding the toxicity of the same chemical to different organisms. Earlier work has shown that N-based organic explosives such as TNT, RDX, and HMX can adversely affect terrestrial ecological receptors (Talmage *et al.,* 1999; Sunahara *et al.,* 2001, reviewed by Sunahara *et al.,* 2009). RDX, a cyclic nitramine, significantly reduced earthworm (*Eisenia andrei*) reproduction at concentrations as low as 95 mg/kg in soil (Robidoux *et al.,* 2000). However, mortality and reproduction of the enchytraeid worm *Enchytraeus crypticus* and the collembolan *Folsomia candida* were not reduced in soils amended with up to 1000 mg RDX/kg soil (Schafer and Achazi, 1999). Furthermore, these studies were conducted either in standard artificial soil (Robidoux *et al.,* 2000), or in soil with relatively high (2.5 - 3.0%) organic carbon (Schafer and Achazi, 1999), which limits their usefulness for development of Eco-SSLs. Simini *et al.* (2003) reported EC_{20} values for cocoon production of 1.2 and 19 mg RDX/kg in freshly amended and weathered-and-aged RDX in Sassafras sandy loam (SSL) soil, respectively. EC_{20} values for juvenile production were 1.6 and 5.0 mg/kg in freshly amended soils and weathered-and-aged soils, respectively. In the same study, the EC_{20} values for HMX freshly amended into soil were 2.7 and 0.4 mg/kg for cocoon production and juvenile production, respectively. Both cocoons and juveniles were not significantly reduced by exposure to weathered-and-aged HMX in soils containing ≤562 mg HMX/kg soil.

As with nitramine EMs, toxicity of nitroaromatic EMs to soil invertebrates has not been sufficiently investigated. The majority of studies focused primarily on the effects of TNT and/or its degradation products (Phillips *et al.,* 1993; Simini *et al.,* 1995; Rocheleau *et al.,* 1999; Schafer and Achazi, 1999; Robidoux *et al.,* 1999, 2000, 2002a,b; Renoux *et al.,* 2000; Sunahara *et al.,* 2001; Dodard *et al.,* 2003; Lachance *et al.,* 2004; Kuperman *et al.,* 2005, 2006a). Dodard *et al.* (2003) reported an EC_{50} value of 111 mg/kg for TNT for juvenile production of *E. albidus*

in *Organisation for Economic Cooperation and Development* (OECD) artificial soil. Phillips *et al.* (1993) reported 100 percent mortality for the earthworm *E. fetida* in USEPA Standard Artificial Soil amended with a mixture of EMs that included 30, 50, 62.5, and 20 mg/kg of TNT, TNB, 2,4-DNT and 2,6-DNT, respectively. Statistically significant ($p < 0.01$) loss of mass was reported at 6, 10, 12.5, and 4 mg/kg of TNT, TNB, 2,4-DNT and 2,6-DNT in soil, respectively. Few studies considered the effects of weathering-and-aging of EM in amended soils on EM toxicity to soil invertebrates. Dodard *et al.* (2003) reported decreased TNT toxicity to *E. albidus* in OECD artificial soil following a 21-d aging period. The EC_{50} for reproduction was 44 and 89 mg/kg in freshly amended and weathered-and-aged TNT in soil, respectively. Specific mechanisms that cause changes in toxicity of EM weathered-and-aged in soil are not known. Lachance *et al.* (2004) showed that the lethality of partially reduced products of TNT, 4-ADNT, and 2-ADNT can be different to *E. andrei* in amended forest soil compared to the parent compound TNT. It is not known if weathering-and-aging EM in soil will influence the toxic effects of these nitroaromatics in earthworms, as suggested by earlier studies using collembola or potworms exposed to 2,6-DNT in SSL soil (Kuperman, 2003; Kuperman *et al*., 2006a).

Our research team recently found the new explosive, CL-20 to be highly toxic to reproduction of earthworm (*E. fetida* and *E. andrei*), potworm (*Enchytraeus crypticus*), and Collembola (*Folsomia candida*). EC_{20} values for earthworm cocoon production and juvenile production were 0.06 and 0.055 mg/kg, respectively (Robidoux *et al.*, 2004c; Simini, unpublished data). Similar severe reproductive toxicities were found for potworms, and Collembola (Kuperman, *et al.*, 2006b; Phillips, unpublished data). CL-20 was more lethal to *E. andrei* (LOEC = 9 mg/kg SSL soil; Robidoux *et al.*, 2004c) than to *E. fetida* (LOEC >500 mg/kg; Simini, unpublished data). These results indicated that CL-20 is more toxic than the traditional nitramine and nitroaromatic explosives.

Simini *et al.* (1995) assessed the toxicity of soil from Joliet Army Ammunition Plant contaminated with a mixture of EMs, including both nitroaromatic and nitro-heterocyclic compounds using earthworm *E. fetida* growth and survival test, among other bioassays. The greatest soil concentrations measured at this site for TNB, 2,4-DNT, and 2,6-DNT were 200, 117, and 8 mg/kg, respectively. Authors reported that TNT and TNB had greatest coefficients of determinations (R^2) in all bioassays, including the earthworm test. R^2 values for TNB using earthworm test endpoints were 0.773 and 0.814 for two locations investigated at the study site. These values for 2,4-DNT were 0.613 and 0.358, whereas 2,6-DNT had the weakest relationship to measurement points with R^2 values of 0.082 and 0.293 for the same two locations, respectively. The weak relationship determined for 2,6-DNT is most likely due to its very low concentrations in soil.

This brief literature review shows that despite considerable attention to assessing ecotoxicity of EMs for soil invertebrates, few studies were designed to specifically meet the USEPA (2005) criteria for derivation of toxicity benchmarks acceptable for Eco-SSL development. The few studies that meet the criteria for Eco-SSL development were conducted by members of our combined research team as part of SERDP-funded projects (CU-1221 and CU-1210), and have produced toxicity benchmarks that were used for development of draft Eco-SSL values for soil invertebrates for both nitramine (Simini *et al*., 2003; Kuperman *et al*., 2003; 2006c), and nitroaromatic EMs (Kuperman *et al*., 2005, 2006b).

2.3. Effects of energetics on soil biological activity

Microbes are essential components of soil systems and are important determinants for soil fertility. Studies have shown that EMs can affect basic soil processes, including carbon and nitrogen cycling (Kuperman *et al*., 2009; Siciliano *et al.*, 2000a,b; reviewed by Sunahara *et al.*, 2009), and that these effects can be reflected in the functional diversity and resiliency of the microbial communities (Fuller and Manning, 1998; Gong *et al.*, 1999a, 2000, 2002; Siciliano and Greer, 2000; Siciliano *et al.*, 2000a, b). Assessment of the soil microbial activity can provide valuable information on the effects of EMs on critical ecosystem-level processes such as energy and nutrient cycling, and complimented our single-species toxicity testing. By including assessment of EM effects on soil microbial activities, we generated data that can provide information on the level of reliability, practicality, and relative sensitivity of microbial assessment endpoints, if used in conjunction with Eco-SSLs within the framework of the SLERA at EM contaminated sites.

2.4. Bioaccumulation assessment

Plant uptake of RDX has been assessed mostly in hydroponic or wetland systems (Harvey *et al*., 1991; Best *et al*., 1999). Recent investigations have demonstrated that RDX and HMX in soil are resistant to aerobic biodegradation (Singh *et al*., 1998), and both tend to accumulate in higher plants (Cataldo *et al.*, 1989; Harvey *et al*., 1991; Best *et al*., 1999; Groom *et al*., 2002). Recent studies showed that HMX can accumulate in plants exposed to HMX amended soils or contaminated field soils (Robidoux *et al.*, 2001a, 2003). Therefore, plant uptake may be an important route for removal of RDX and HMX from soil. Conversely, bioaccumulation of these two EMs may significantly impact higher trophic-level receptors via transfer through the food chain. HMX may also accumulate in earthworms exposed to HMX amended soils or contaminated field soils (Robidoux *et al.*, 2001b, 2004a, b). Transformation products of the nitroaromatics TNT and DNT have been found in earthworms and enchytraeids exposed to soils amended with EMs (Renoux *et al*., 2000; Johnson *et al.*, 2000; Robidoux *et al*. 2002b; Lachance *et al.*, 2003, 2004; Dodard *et al.*, 2004; reviewed by Sunahara *et al.*, 2009). Our research team investigated bioaccumulation of RDX and HMX in soil invertebrates using radiolabeled compounds (SERDP CU-1221). Lachance *et al.* (2003) showed that ^{14}C-RDX or ^{14}C-HMX was accumulated by three plant species, and most of the radiolabeled RDX or HMX was not metabolized. Studies with earthworm using ^{14}C-RDX or ^{14}C-HMX produced relatively low bioaccumulation factors. The RDX concentration ratios in earthworm tissue and soil (BAF) decreased from 13 to 2.9 as nominal RDX concentrations in soil increased from 10 to 100 mg/kg. Statistical analysis of these data revealed a log-linear relationship between RDX concentrations in soil and in earthworms (Tsao *et al.*, 2005), with a median BAF value of 2.6 (Tsao and Sample, 2005). Alternatively, Best *et al.* (2006) reported an average BAF=1, based on a 28-d study using *E. fetida* exposed to a field-collected soil containing RDX and other contaminants, including HMX and 1,3-dinitrobenzene. Results of the latter study would indicate that RDX has little to no bioaccumulation potential. It was not reported by those authors whether the presence of the HMX or dinitrobenzene had an effect on RDX accumulation. Therefore, there are a number of discrepancies in the literature describing the soil bioaccumulation of RDX, as reflected by the various BAF values reported.

These inconsistencies can be attributed to differences in experimental protocols used to measure bioaccumulation, including the earthworm species, soil types, RDX concentrations and ranges, duration of exposures, presence of co-contaminants in field-collected soils, as well as formation of RDX transformation products in soil or in the organism.

The ER-1416 project directly addressed the knowledge gap concerning the bioaccumulation potential of 2,4-DNT, HMX, NG, TNT, and RDX directly from soil by terrestrial plants, and earthworms. Bioaccumulation of these EMs was assessed for plants and earthworms using unlabeled materials as well as radiolabeled EM compounds. The use of ^{14}C-labeled EMs lessened analytical problems associated with interference from other organic compounds that are present in soil and tissue samples.

Soil EM exposure is often analytically determined using an organic solvent extract of EM-contaminated soil. However, it is not known to what extent such measurement reflects the bioavailability of moderately hydrophobic EMs in a soil matrix. Assessment of EM bioaccumulation and metabolism by higher plants and soil invertebrates using ^{14}C-compounds has allowed us to better understand and quantify bioavailability as well as the biotic and abiotic fate pathways of these EMs. Measurement of different fractions (organic solvent-extractable vs. non-extractable) of the ^{14}C-EMs in soil also enabled us to relate certain “bioavailable” fractions of these compounds to their observed toxicities by establishing concentration-response relationships.

3. Materials and Methods

3.1. Experimental design

Studies were designed to generate toxicity benchmarks that will be acceptable for development of Eco-SSLs for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG for terrestrial plants, and soil invertebrates. In addition, bioaccumulation of 2,4-DNT, HMX, NG, TNT, and RDX was investigated using an integrated ecotoxicology and chemistry approach. This project required that toxicity and bioaccumulation studies be carried out in parallel with chemical analyses to link the environmental behavior of EMs with their respective ecotoxicities. The general experimental approach is shown in Figure 1.

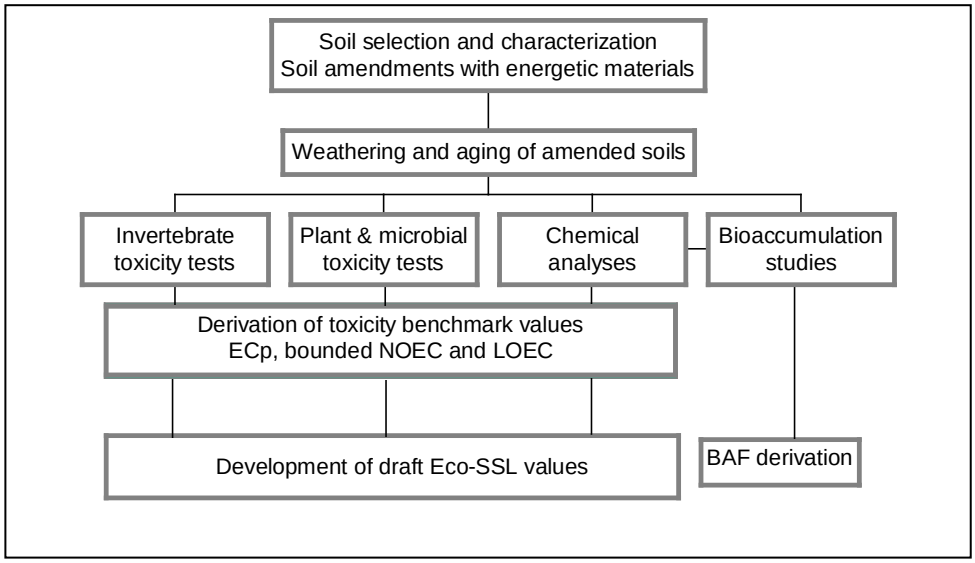


Figure 1. Overview of the technical approach.

3.2. Test soils collection, preparation, and characterization

Studies were conducted using soils with a relatively wide range of physico-chemical characteristics. These soils included Teller sandy loam [Fine-loamy, mixed, active, thermic Udic Argiustoll] (TSL; collected from agricultural land of the Oklahoma State University Perkins Experiment Station, Payne county, OK), Sassafras sandy loam [Fine-loamy, siliceous, semiactive, mesic Typic Hapludult] (SSL; collected from an open grassland field in the coastal plain on the property of the U.S. Army Aberdeen Proving Ground, Harford County, Maryland), Kirkland clay loam and Kirkland loam [Fine, mixed, superactive, thermic Udertic Paleustoll] (KCL and KL; collected in Payne county, Oklahoma), and Webster clay loam [Fine-loamy, mixed, superactive, mesic Typic Endoaquoll] (WCL; collected in Story country, Iowa). During soil collection in the field, vegetation and the organic horizon were removed and the top 12 cm of the A-horizon were then collected. Soil was sieved through a 5-mm mesh screen, air-dried for at least 72 h and mixed periodically to ensure uniform drying, passed through a 2-mm sieve, then

stored at room temperature before use in testing. Soil was then analyzed for physical and chemical characteristics. The qualitative relative bioavailability (QRB) scores for organic chemicals in natural soils were considered “very high” for TSL and SSL, and “medium” for KCL, KL, and WCL soils, according to the Eco-SSL criteria (USEPA, 2005). Results of these analyses are presented in Table 1. The SSL and TSL soils had sufficiently low organic matter and clay contents to fulfill the USEPA requirement for using soils with characteristics that support high relative bioavailability of organic pollutants, for developing realistic conservative Eco-SSL values (USEPA, 2005). Although ecotoxicological data determined in these soils can be representative of potential exposure effects in soils with similar chemical bioavailability conditions, such data can overestimate or underestimate the toxicities of EM in soil types with properties that contrast with those of SSL or TSL. Therefore, studies with additional natural soils (KCL, KL, and WCL) representing a range of soil parameters were conducted with 2,4-DNT to extend the range of soil physico-chemical characteristics, hypothesized to affect EM toxicity (USEPA, 2005), in order to ascertain the relationships among predominant soil physico-chemical parameters and the toxicity of 2,4-DNT for plants, and soil invertebrates.

3.3. Test energetic materials

Energetic materials used in this investigation included nitroaromatics 2,4-dinitrotoluene (2,4-DNT; CAS: 121-14-2; Purity: 97%), 2-amino-2,6-dinitrotoluene (2-ADNT; CAS 35572-78-2; Purity 99%), 4-amino-2,6-dinitrotoluene (4-ADNT; CAS 19406-51-0; Purity 99%), 2,4,6-trinitrotoluene (TNT; CAS 118-96-7; Purity 99%), and nitroglycerin (NG; CAS: 55-63-0; purity: 99%), as well as nitramines octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX; CAS: 2691-41-0; Purity: 99%), and hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX; CAS: 121-82-4; Purity: 99%). All EMs tested were obtained from the Defense Research Establishment Valcartier of the Canadian Ministry of National Defense (Val Bélair, QC, Canada).

3.4. Soil amendment procedure for toxicity tests

Prepared soils were weighed separately in a glass container for each EM treatment, and were spread from 2.5 to 4 cm thickness. Each EM amendment was prepared separately in a glass volumetric flask and dissolved in acetone as carrier, to produce a more homogeneous mixture in soil than the addition of solid EM chemicals. Each soil was individually amended with the selected EM. The EM/acetone solution was quantitatively transferred to the soil, adding it evenly across the soil surface, ensuring that the volume of solution added at any one time does not exceed 15% (v / w) of the dry mass soil. The same total volume of acetone was added to every EM treatment, equaling the volume of acetone required to dissolve the EM at the highest concentration tested. The amended soils were then air-dried for a minimum of 18 h in a darkened chemical fume hood. Each amended soil sample was transferred into a high-density polyethylene container coated with a fluoro-polymer, mixed for 18 h using a three-dimensional soil mixer in darkness to prevent photolysis of the EM. Seven to nine concentrations of each EM were used in addition to controls (negative, positive, and carrier). All treatments were appropriately replicated. After three-dimensional mixing, samples of freshly amended soil were collected from each soil treatment batch and were sent overnight to BRI for analytical determinations of the initial EM concentrations using USEPA Method 8330A (USEPA, 2007). The remaining soil in each batch

was hydrated with ASTM type I water to 60% of the respective soil's water holding capacity (WHC) initiating the EM weathering-and-aging procedure in soil.

3.5. Weathering-and-aging of energetic materials in soils for toxicity tests

Special consideration in assessing EM toxicity for Eco-SSL development was given to the inclusion of weathering-and-aging of contaminant explosives in soil in the assessment of the EM effects on terrestrial receptors. This more closely approximates the exposure effects in the field, and is more relevant for Ecological Risk Assessment (ERA) because Eco-SSL development by USEPA was specifically undertaken for use at Superfund sites (locations where contaminants have been long-present). Weathering/aging of chemicals in soil may reduce exposure of terrestrial plants and soil invertebrates to EMs due to photodecomposition, hydrolysis, reaction with organic matter, sorption/fixation, precipitation, immobilization, occlusion, microbial transformation, and other fate processes that commonly occur at contaminated sites. This can result in a dramatic reduction in the amount of parent compound that is bioavailable, compared to tests conducted with recently-amended chemicals or those tested following a short equilibration period (*e.g.*, 24 h), and can affect the EM toxicity as was demonstrated in our previous studies. Standardized methods for weathering/aging of EM-amended soil are not available. We have developed approaches that simulate, at least partially, the weathering and aging process of chemicals in field soil (Kuperman *et al*., 2004a,b; 2005; 2006b).

The weathering-and-aging procedure of EMs was performed at U.S. Army Edgewood Chemical Biological Center (ECBC) in preparation for the definitive toxicity testing with terrestrial plants and soil invertebrates. It was conducted to simulate, at least partially, the weathering-and-aging process in field soils, and to more closely approximate the exposure effects on soil biota at contaminated sites. These procedures included exposing amended and control soils, initially hydrated to 60% of the WHC of each soil type, in open glass containers in the greenhouse at ambient temperature to alternating moistening and air-drying cycles for three months. NG was weathered-and-aged in soil for one month because of its rapid degradation in hydrated soil, as described in Section 4.1.10. During the weathering-and-aging procedure, all soil treatments were weighed and readjusted to their initial mass by adding ASTM type I water to the soil each week. Soil samples collected from each treatment after the three-month weathering-and-aging procedure, which corresponded to the beginning of the definitive toxicity tests, were sent overnight to BRI for analytical determinations of EM concentrations.

3.6. Soil amendment procedure for microbial activity tests

Sassafras sandy loam (SSL2007e, Table 1) soil, similar to that used in the standardized single-species soil invertebrate and terrestrial plant toxicity tests (SSL2007d), was used to assess soil microbial activity endpoints. During soil collection, the root zone of the upper soil layer was retained to ensure sufficient abundance of the indigenous soil organisms. Fresh SSL2007e soil was collected in November 2007 from the same location on the coastal plain of the U.S. Army Aberdeen Proving Ground (APG) in which SSL2007d soil was collected. Soil was gently passed through a 5-mm sieve to remove large debris and regularize distribution of soil organisms, then stored in covered plastic containers overnight to preserve the initial field moisture level. A portion of previously collected SSL soil (SSL2007d), set aside for subsequent preparation of EM

soil concentrates, was treated by prolonged heating (three days after constant mass was achieved) at 80°C to minimize potential introduction of additional organisms present in this soil to the overall biological activity (combined microbial and micro-invertebrate communities) in the final soil treatments of EMs. This heat-treated SSL2007d soil was then sieved through a 2-mm sieve, and used to prepare EM soil concentrates.

Soil concentrates of individual EM were prepared to uniformly amend into fresh field-moist SSL2007e soil, with target treatment concentrations of 10, 100, 1000, and 10000 mg/kg. EM was amended into treatment soils in the form of EM soil concentrates in order to avoid harming soil organisms by exposure to carrier solvent (acetone). Soil concentrates of individual EMs were prepared in three steps. In the first step, concentrates of 1000 (Concentrate I) or 10000 (Concentrate II) mg/kg were prepared by adding 0.1605 or 1.5002 g of crystalline EM to 60 mL of acetone (in three consecutive additions of 20 mL aliquots), then pipetting the respective EM/acetone mixtures onto 159.8 or 148.5 g batches of SSL2007d soil. A separate EM concentrate (Concentrate III) was prepared for the target 10,000 mg/kg treatment by adding 14.0045 g of crystalline EM to 60 mL of acetone soil, and then pipetting the EM/acetone mixture onto 126 g of SSL2007d soil. The acetone was allowed to volatilize overnight in a fume hood in darkness. Amended SSL2007d soil batches were then mixed for 18 h using a three-dimensional rotary soil mixer. In the second step, an intermediate EM concentrate (Concentrate IV) was prepared for the target 10 mg/kg treatment by adding 14 g of EM Concentrate I to 126 g of SSL2007d soil, then mixing the components for 18 h using a three-dimensional rotary soil mixer. The final target EM treatments were prepared in the third step. Target 10 mg/kg treatment was prepared by adding 140 g of Concentrate IV to 1260 g of fresh SSL2007e soil. Target 100 or 1000 mg/kg treatments were prepared by adding 140 g of EM Concentrates I or II, respectively to 1260 g of fresh SSL2007e soil. Target EM treatment of 10000 mg/kg was prepared by adding 140 g of Concentrate III to 1260 g of fresh SSL2007e soil. All treatments were prepared one day after collecting SSL2007e soil in the field by individually combining and gently mixing EM soil concentrates with clean SSL2007e soil in separate plastic bags. The carrier (acetone) control treatment was prepared by adding 140 g of Concentrate 0 to 1260 g of fresh SSL2007e soil. This approach ensured that the amount of fresh SSL2007e soil containing indigenous organisms remained constant throughout the range of treatments. The field soil moisture level of 14.4% dry soil mass at the time of soil collection was maintained for the duration of the study by weekly additions of ASTM type I water. Soil samples were collected from each EM treatment and carrier controls for analytical determination of EM concentrations. These samples were frozen at -80°C and then sent to BRI for analyses.

3.7. Analytical measurement of energetic materials in soil

At the beginning of each toxicity test, concentrations of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG were analytically determined using a modified USEPA Method 8330A (USEPA, 2007). A 2-g soil sample was collected from each soil batch, placed into a 50-mL glass tube, and 10 mL acetonitrile was added to the tube. Internal standards were added (100 μL) to each tube to evaluate the extraction efficiency. Internal standards were 1,3-DNB for 2,4-DNT, 2-ADNT, and 4-ADNT; 2,4-DNT for HMX; and HMX for NG. Soil extraction was repeated if internal standard recovery was less than 90%. Samples were then vortexed for 1 min and sonicated in darkness for 18 h at 20°C. After letting the sonicated samples settle for 1 h at room temperature,

5 mL of supernatant was transferred to a glass tube, to which 5 mL of $CaCl_2$ solution (5 g/L) was added as a flocculent. Supernatant was filtered through a 0.45 µm polytetrafluoroethylene syringe cartridge. One mL of this filtered solution was transferred to an HPLC vial. Soil extracts were analyzed and EM concentrations quantified using a Waters HPLC system composed of a Model 600 pump, a Model 717 Plus injector, a Model 2996 photodiode-array, and a temperature control module. Calibration curves were generated before each HPLC run using certified standards (AccuStandard, New Haven, CT or Cerilliant, Round Rock, TX) of each EM, in a range of concentrations appropriate for each set of determinations. The limits of detection were 0.01, 0.005, 0.005, 0.034, and 0.05 mg/L for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG, respectively, corresponding to 0.1, 0.05, 0.05, 0.34, and 0.5 mg/kg (dry soil mass).

3.8. Analytical measurement of energetic materials in earthworms

The quantification of all EM, including RDX, HMX, 2,4-DNT, TNT, and NG, in earthworm tissue was done using procedure described in Renoux et al. (2000) and Sarrazin et al. (2009). Briefly, depurated earthworms were rinsed, blotted-dry, placed into glass tubes, and were immediately frozen at –80°C. For each replicate, six earthworms were lyophilized, then crushed using a mortar and pestle to obtain approximately 500 mg dry material for analysis. Two mL of ASTM type I water (4°C) was added to each tissue sample followed by vortexing for 10 sec. Five mL of acetonitrile was then added to the suspension, which was then vortexed for an additional 60 sec. All samples were then sonicated (Branson Model 3200, Danbury, CT) for 18 $\pm$ 2 h at 20°C, and centrifuged (12000 x g) for 10 min at 4°C using a Sorval Super T21 centrifuge (Global Medical Instrumentation, Ramsey, MN). A 3.5 mL-aliquot of each supernatant was treated with 1.5 mL of $CaCl_2$ (16 g/L) and placed at 4°C for 2 h in order to precipitate fine particles. The supernatant was filtered through a 0.45 µm Millex-HV cartridge before chemical analysis. One mL of the filtrate was transferred to an HPLC vial. EM concentrations were quantified by reversed-phase HPLC using a modified USEPA Method 8330A (USEPA, 2007). Calibration curves were generated before each HPLC run using certified standards (AccuStandard, New Haven, CT or Cerilliant, Round Rock, TX) of each EM, in a range of concentrations appropriate for each set of determination. The HPLC limits of detection were 0.032, 0.034, 0.01, 0.005, and 0.05 mg/L for RDX, HMX, 2,4-DNT, TNT, and NG, respectively. The limits of quantification in tissue extracts were 5.0 µg/g for RDX or HMX, 2 µg/g for 2,4-DNT, 4 µg/g for TNT, and 1.5 µg/g for NG.

3.9. Analytical measurement of energetic materials in plants

All plant tissues, except NG exposed tissues, were lyophilized and were extracted dry. Plant tissues exposed to NG were extracted wet to avoid volatilization of potential NG metabolites. Lyophilized plant tissues (shoots or roots) were ground using mortars and pestles, then transferred to a glass conical tube and 500 µL of internal standard solution was added to each tube. All internal standards were prepared in acetonitrile to reach a maximum concentration of 1 mg/L. Wet tissue was homogenized using a Dounce tissue grinder and 1 mL of internal standard: $CaCl_2$ (5 g/L) solution (1:1; v:v). The Dounce was rinsed twice with 0.5 mL of the internal standard: $CaCl_2$ solution. Homogenate was transferred to a centrifugation tube. Internal standards used to verify extraction efficiency were RDX for HMX; TNT for 2,4-DNT; HMX for RDX;

2,4-DNT for TNT; and RDX for NG. Samples were then vortexed for 1 min, sonicated for 18 ± 2 h at 20°C, and centrifuged (Allegra X-12R, Beckman Coulter) at 1500 rpm for 1 h. For lyophilized plant tissues, a 400-µl aliquot of supernatant was transferred into a glass vial, to which an equivalent volume of $CaCl_2$ (5 g/L) was added. For wet plant tissues, a one-mL aliquot of supernatant was transferred into a glass vial. Samples were vortexed and kept at 4°C for 24 h. Supernatants were filtered through 0.45 µm cartridges to remove fine particles. 150 µl of this filtered solution was transferred to an HPLC vial fitted with an insert. Individual EM concentrations were quantified by reversed-phase HPLC using a modified USEPA Method 8330A (USEPA, 2007). Calibration curves were generated before each HPLC run using certified standards (AccuStandard, New Haven, CT or Cerilliant, Round Rock, TX) of each EM, in a range of concentrations appropriate for each set of determinations. The HPLC limits of detection were 0.032, 0.034, 0.01, 0.005, and 0.05 mg/L for RDX, HMX, 2,4-DNT, TNT, and NG, respectively. The limits of quantification in the root or shoot extracts were 5.1, 5.4, 2.3, 1.6, and 8.0 mg/kg (dry tissue).

3.10. Terrestrial plant toxicity tests

Plant toxicity tests were performed according to ASTM (2002) and USEPA (1996) standard protocols using alfalfa *Medicago sativa*, barnyard grass *Echinochloa crusgalli* and perennial ryegrass *Lolium perenne,* variety Express. Twenty seeds of each plant species were separately sown in each 10-cm pot containing 200 g of dry soil. The bottom of each plant pot was previously covered with a piece of cheesecloth to prevent soil loss during testing. Alfalfa seeds were inoculated with endosymbiotic nitrogen-fixing Rhizobium bacteria prior to sowing. ASTM type I water (negative control) or boric acid solution (positive control) was added to obtain 75% of the soil WHC. Concentrations of boric acid (H_3BO_3) tested as positive control were 175, 200, 230, 260, and 290 mg/kg for alfalfa; 65, 110, 175, 260, 350, and 450 for barnyard grass; and 50, 80, 110, 150, and 200 mg/kg for ryegrass. Plant pots were placed in 1-L polyethylene bags closed with an elastic band to minimize loss of soil water due to evapo-transpiration. Plant toxicity tests were performed in a temperature and light controlled growth chamber. Plants were incubated in darkness for the first two days and then exposed to a diurnal photoperiod cycle afterwards. The growth chamber conditions were set as follows: light intensity at 5000±500 lux, light for 16 h at 25°C, dark for 8 h at 20°C. Luminosity level was measured weekly using a photometer, and the light intensity was readjusted when needed. The measurement endpoints included seedling emergence, shoot wet mass, and shoot dry mass. Seedling emergence was measured after 5 d for alfalfa and barnyard grass, and after 7 d for ryegrass. Shoot growth was measured after 16 d alfalfa and barnyard grass, and after 19 d for ryegrass. Plants dry mass was determined by drying the tissue at 70°C for 24 h.

3.11. Soil invertebrate toxicity tests

3.11.1. Earthworm toxicity tests

Earthworms (*Eisenia fetida* Savigny 1826) were bred in plastic containers filled with approximately 14 kg of a 1:1 mixture of sphagnum PRO-GRO peat moss (Gulf Island Peat Moss Co., PEI, Canada) and BACCTO® potting soil (Michigan Peat Co., Houston, TX, USA). The pH was adjusted to 6.26 ± 0.07 by adding calcium carbonate (pulverized lime). The culture was kept

moist at 21 ±2°C with continuous light**.** Earthworm colonies were fed biweekly with dehydrated alfalfa pellets (27% fiber, 17% protein, 1.5% fat; OB of PA, York, PA) that prior to feeding were fermented, dried, and ground to a course powder. Cultures were synchronized so that all worms used in each test were approximately the same age.

The Earthworm Toxicity Tests were conducted according to ISO/11268-2 (ISO, 1998a) to assess the effects of test chemicals on the *E. fetida* adult survival and reproduction endpoints. Adult earthworms (five per replicate container) with fully developed clitella, and weighing from 0.3 to 0.6 g were randomly selected and placed in 550 cc glass containers filled with 200 g of test soil. Two grams of prepared worm food (see above) were placed in each container. A piece of clear plastic film was stretched over the top of each container and secured in place with a screw top. Three pinholes were made in the plastic film to facilitate air exchange. Four replicates were prepared for each treatment and controls (negative and positive). The mass of each test containers was recorded. All containers were placed in an environment-controlled incubator under a 16 h-light:8 h-dark photoperiod cycle with a mean photosynthetically active radiation (PAR) light intensity of 12.8±0.7 µM/m/sec (985±52 lux), mean relative humidity of 86±2%, and mean temperature of 21.6±0.1°C. Adults were removed and counted after 28 days. Juveniles and cocoons were removed and counted after 56 days from the start of exposure.

Toxicity tests with reference toxicant boric acid (positive control) were conducted throughout the project using SSL soil to assess changes in sensitivity, health, and performance of *E. fetida* maintained in ECBC laboratory cultures. Test treatments were prepared by adding appropriate solutions of boric acid in ASTM type I water to SSL soil. Regression analyses of toxicity data from independent studies were used to establish the respective EC_{50} values and corresponding 95% Confidence Limits (CL) for adult survival and production of juveniles. These values were plotted on a Boric Acid Warning Chart using modified procedures described in a previous report (EC, 2005). The modification included using calculations based on arithmetic (untransformed) EC_{50} values for boric acid concentrations instead of logarithmic concentrations.

Validity criteria were included in testing as part of the Quality Control procedures. They included the following performance parameters for the negative controls:

1) The mean adult mortality does not exceed 10% in range-finding or definitive tests;
2) The number of juveniles per five worms is ≥15; and
3) The coefficient of variation for production of juveniles is ≤50% at the end of the test.

3.11.2. Potworm toxicity tests

The Enchytraeid Toxicity Test was used to assess the effects of selected EM on the enchytraeid worm (potworm) *Enchytraeus crypticus* (Westheide & Graefe 1992). The test is an adaptation of an International Organization for Standardization (ISO) bioassay ISO/16387 *Soil quality — Effects of pollutants on Enchytraeidae (Enchytraeus sp.) — Determination of effects on reproduction and survival* (ISO, 2004b). This test was selected on the basis of its ability to measure chemical toxicity to ecologically relevant test species during chronic assays, and its inclusion of at least one reproduction component among the measurement endpoints. The ISO Guideline for this assay was originally developed for use with Organization for Economic Co-

operation and Development (OECD, 1984) artificial soil (similar soil formulation was later adapted for USEPA Standard Artificial Soil, SAS; USEPA, 1996; and for ASTM Artificial Soil, AS; ASTM E1676-04, 2004). However several studies demonstrated that this test could also be conducted using natural soils (Amorim et al., 2009; 2005a,b; Dodard et al., 2005; Kuperman *et al.*, 2003; 2004a,b; 2005; 2006a-d). The ISO/16387 was initially developed using the enchytraeid worm species *E. albidus.* Results of our previous studies using *E. albidus* showed that this species requires soils containing high organic matter content with a soil pH 6 (±0.5) for optimal test conditions. This species performed poorly in natural soils having physical and chemical characteristics that support a higher level of EM bioavailability (Amorim et al., 2009; 2005a; Kuperman *et al.*, 1999; 2006a). The species of genus Enchytraeidae, *E. crypticus*, listed in the ISO protocol as an acceptable alternative to *E. albidus*, was selected for toxicity testing.

Potworms were bred in 4.3-L clear plastic boxes (34 x 20 x 10 cm) filled with 2 kg (dry mass) SSL soil. The culture was kept in an environment-controlled incubator under a 16 h light, 8 h dark photoperiod cycle, with a mean photosynthetically active radiation (PAR) light intensity of 12.8 ± 0.7 (SE) µM/m/sec (985 ± 52 lux) and mean temperature of 21.6 ± 0.1°C. Soil moisture level was adjusted to 100% of the WHC of SSL soil, and was maintained by periodic (once per week) mass checks and water adjustments. Soil in the breeding culture was aerated by carefully mixing it once each week. The potworms were fed approximately twice each week with ground oats spread onto the soil surface. If food from the previous feeding date remained on the soil surface, the amount of food added was adjusted. Every 6 weeks, the worms were transferred into a freshly prepared culture substrate. Cultures were synchronized so that all worms used in each test were approximately the same age. The potworm culture was considered healthy if worms were whitish in color, reproduced continuously, did not try to leave the soil, and exhibited a shiny outer surface with no soil particles clinging to them.

Glass jars (42 mm ID; 45 mm deep) were used as test containers. They were rinsed with acetone, tap water, and ASTM type I water before the tests. Twenty grams (dry mass basis) of test soil and 0.05 g of ground oats were added to each test container, then mixed and hydrated to 100% of the WHC of each soil. The mass of each container with soil was recorded.

Adult potworms with eggs in the clitellum region were used for testing. They were collected from culture and were placed in a Petri dish filled with a small amount of ASTM type I water for examination using a stereomicroscope. Potworms with no eggs were discarded. Any invertebrates living in the cultures (such as mites) were also removed. Ten potworms selected for uniformity (approximately 1 cm in length) were placed on top of the soil in each test container. Plastic wrap was stretched over the top of each container and secured with a rubber band. Three pinholes were made in the plastic wrap to facilitate air exchange. All containers were placed in an environment-controlled incubator under the same conditions as described above for maintenance of the potworm culture. The containers were weighed once a week and the mass loss was replenished with the appropriate amount of ASTM type I water. Ground oats (0.05 g) were added to each test container at that time.

After two weeks, soil in each test container was carefully searched and adult potworms were removed and counted. Potworms were examined for any morphological or behavioral changes. The remaining test substrate, including any cocoons laid during the first two weeks of the test, was incubated for an additional two weeks. After four weeks from the start of the test, soil in the test containers was fixed with 70% ethanol, and nine drops of Rose Bengal biological stain (1% solution in ethanol) were added. Staining continued for minimum of 24 h. The content of each

test container was wet-sieved using a No. 100 mesh sieve (150 µm), and retained contents transferred to a counting tray where potworms were counted. Measurement endpoints included number of surviving adults after 14 days and number of juveniles produced after 28 days.

Treatment concentrations for definitive tests with each EM were selected based on the results of the range-finding tests to bracket the 20% and 50% inhibition in production of juveniles, compared with production of juveniles in carrier control for each soil. Based on the results of previous tests with SSL soil, the Limit Test was conducted to assess the effects of HMX weathered-and-aged in TSL soils on adult survival and production of juveniles by *E. crypticus*. The Limit Test is a variant of the definitive test, and is performed when statistical analysis of the range-finding test data show no significant differences among all treatment concentrations of a test chemical. All definitive tests included negative controls (no chemicals added) and carrier (acetone) controls.

Toxicity tests with reference toxicant boric acid (positive control) were conducted throughout the project using SSL soil to assess changes in sensitivity, health, and performance of *E. crypticus* maintained in ECBC laboratory cultures. Test treatments were prepared by adding appropriate solutions of boric acid in ASTM type I water to SSL soil to obtain nominal concentrations of 0 (negative control), 20, 30, 50, 80, 100, and 200 mg/kg. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective EC_{50} values and corresponding 95% CL for juvenile production. These values were plotted on a Boric Acid Warning Chart, using modified procedures described by Environment Canada (EC, 2005), in order to monitor the condition of the potworms and precision within laboratory culture. The modification included using calculations based on arithmetic (untransformed) EC_{50} values for boric acid concentrations instead of logarithmic concentrations.

The following replication was used in the definitive tests: four replicates of each EM treatment or control for the definitive tests with multiple treatment concentrations. Replication in the Limit Test included eight replicates in 0 mg/kg (carrier control), eight replicates in 10,000 mg/kg (the greatest nominal HMX concentration selected for study with TSL soil), and four replicates in the negative control. Validity criteria for the negative controls in all toxicity tests included the following performance parameters (ISO/16387, 2004):

1) The adult mortality does not exceed 20% after 14 days;
2) The average number of juveniles is greater than 25 per test container at the end of the test, assuming that 10 adult worms per test container were used;
3) The coefficient of variation for the mean number of juveniles is ≤50%

3.11.3. Collembola toxicity tests

The culture of *Folsomia candida* Willem 1902 (Collembola) was maintained in culture jars on a mixture of charcoal and plaster of Paris in darkness at approximately 20°C. The collembolans were fed baker's yeast and kept moist by routine misting with ASTM type I (ASTM, 2004) water approximately twice per week. Synchronized cultures were established for the experiments by removing egg clusters from stock cultures and placing them into new jars. Eggs were monitored daily to determine the onset of hatching. Once hatching began, it was allowed to proceed for 2 d, after which juveniles were transferred to new jars. These synchronized juveniles were then held for 10 d, thus producing the 10-12 day-old juveniles used in tests.

The Folsomia Toxicity Tests were used to assess the effects of test chemicals on the adult survival and reproduction of the collembolan *F. candida*. The Folsomia Toxicity test is an adaptation of an ISO bioassay ISO/11267 (ISO, 1998b). The guidelines for this assay were originally developed for use with Artificial Soil (OECD/USEPA Standard Artificial Soil), however, previous studies have shown that this test can also be conducted using natural soil types. The measurement endpoints of this test included the number of initial juveniles surviving to adulthood and the number of viable offspring produced by *F. candida*.

Glass jars (42 mm ID; 45 mm deep) were used as test chambers for toxicity testing. The jars were rinsed successively with acetone, tap water, and ASTM type I water, then inverted and allowed to air dry. A 100-g soil batch from each EM treatment level was hydrated with ASTM Type I water to 88% of the soil water holding capacity (WHC) on the first day of a test. One-fifth of each batch was weighed and transferred into a test chamber. Baker's yeast (0.05 g) was then added to the surface of the soil in each test chamber. Ten to 12-day-old juveniles were placed in each test chamber, followed by light misting with ASTM type I water. A piece of clear plastic film was placed on each test chamber, held in place with a rubber band, and the film was then perforated with three pinholes to facilitate air exchange. Five replicates were prepared for each treatment and controls (negative and positive). The mass of each test chamber was recorded. The test chambers were placed randomly in an environment-controlled incubator under a 16 h light:8 h dark photoperiod cycle, with a mean photosynthetically active radiation (PAR) light intensity of 12.8±0.7 µM/m/sec (985±52 lux), mean relative humidity of 86±2%, and mean temperature of 21.6±0.1°C. Individual test chambers were weighed once a week, and mass loss was replenished with the appropriate amount of ASTM type I water.

The test was terminated on day 28 of exposure, by adding approximately 15 mL of tap water to each test chamber. The chambers were gently mixed with a spatula, and allowed to sit for several minutes to fully hydrate the soil. An additional 10 mL of water was added to each test chamber, and again mixed with a spatula. The contents of each test chamber were examined under a dissecting microscope (15x) for the presence of adults and juveniles. Total numbers of adults and juveniles, respectively, that floated to the surface were counted and recorded.

Toxicity tests with reference toxicant boric acid (positive control) were conducted throughout the project using SSL soil to assess changes in sensitivity, health, and performance of *F. candida* maintained in ECBC laboratory cultures. Test treatments were prepared by adding appropriate solutions of boric acid in ASTM type I water to SSL soil to obtain nominal concentrations of 0 (negative control), 20, 30, 50, 80, 100, and 200 mg/kg. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective EC_{50} values and corresponding 95% CL for juvenile production. These values were plotted on a Boric Acid Warning Chart, using modified procedures described by Environment Canada (EC, 2005), in order to monitor the condition of the collembolans and precision within laboratory culture. The modification included using calculations based on arithmetic (untransformed) EC_{50} values for boric acid concentrations instead of logarithmic concentrations.

Validity criteria were included in the testing as part of the Quality Control procedures. They included the following performance parameters for the carrier controls:

1) The adult mortality does not exceed 30% after the 28-d test;
2) The average number of juveniles is greater than 80 at the end of the 28-d test;

3) The coefficient of variation for the mean number of juveniles is ≤30% at the end of the test.

3.12. Soil biological activity tests

Toxicological benchmarks for soil functional endpoints were based on internationally accepted standardized toxicity assays, including carbon mineralization assays (ISO, 1997a, 2002; OECD, 2000b; USEPA, 1987), organic matter decomposition assay (EC, 2002; OECD, 2006), and on the methods used routinely in basic and applied soil ecological research, including soil enzyme activity assays (ISO/14238, 1997, ISO/15685, 2004; ISO/22939, 2010 and ISO/23753-2, 2005b). All toxicity benchmarks were developed using freshly collected soil, and were based on the measured EM concentrations.

Fresh SSL (SSL2007e and SSL2011) soil for use in studies requiring living soil biota (invertebrate and microbial communities) was collected from a grassland field on the property of APG, and prepared for studies as described in section 3.6. Soil samples from each treatment were analyzed by HPLC using USEPA Method 8330A (2007) to determine acetonitrile-extractable EM concentrations. All treatments were appropriately replicated and included carrier controls.

3.12.1. Litter decomposition assay

Litter decomposition is one of the most integrating processes within the soil ecosystem because it involves complex interactions of soil microbial and faunal activity with the soil chemical environment (Wentsel *et al*., 2003; Kuperman *et al*., 2002). Any disturbance, which alters organic matter decomposition, can result in nutrient losses and a decline in soil fertility. Therefore, an assessment of how soil contamination with EMs may alter rates of organic matter decomposition is critical to understanding potential EM impact on overall ecosystem structure and function.

Litter decomposition in SSL soil treatments was quantified using Orchard grass (*Dactylis glomerata*) straw. Approximately 200 g of EM-amended soil or carrier control soil was loosely packed into individual test containers (glass jars 900 mL volume, 90 mm diameter). A pre-weighed cluster of three 5-cm long internodular sections of straw was placed on the soil surface in each test container to assess the potential effects of individual EMs on litter decomposition. All containers were placed randomly in an environment-controlled incubator at 22±1^{o}C, 86% relative humidity, and 16:8 h light/dark photoperiod cycle. ASTM type I water was added weekly in order to maintain the initial soil moisture level.

A set of four randomly selected replicates from within each treatment was harvested after 1, 2, 3, 4, 6, and 8 months until approximately 80% mass loss was recorded in the treatment with the greatest decomposition rate. Grass litter was removed and processed to measure mass loss. Annual decomposition rate constants (k) for litter residues, and corresponding standard errors (SE) and regression coefficients (r^2) were determined using the single exponential decay model $m_t/m_0 = e^{-kt}$, where m_t/m_0 = fraction mass remaining at time t, t = time elapsed in years, and k = the annual decomposition constant (Kuperman, 1999). The model was fit to the data by least squares regression of the natural logarithm of mean percent mass remaining over time.

3.12.2. Soil respiration assays

Batches of SSL2011 soil, for basal respiration (BR) and substrate-induced respiration (SIR) assays, were separately amended with individual EM or acetone only (carrier control) as described in Section 3.6. For BR assays, each soil batch was hydrated to about 60% of the soil WHC by adding ASTM type I water to the soil and mixing the soil for 20 seconds. Replicate (n=3) soil samples of 50 g (dry soil basis) were weighed into Soil Incubation Chambers (SIC; 250 mL glass bottles). The SIC were connected to the pre-calibrated Micro-Oxymax respirometer (Columbus Instruments) within 2 h of the soil amendment. The CO_2 evolution rates for BR assays were recorded by the computer software (Columbus Instruments) in 16-h intervals on days 0, 1, 2, 3, 4, 5, 6, 14, and 28. The data collected on day 28 were used to estimate the EC values for each EM.

Prior to SIR assays, the maximum initial respiratory response to glucose was determined for the SSL2011 soil using glucose concentrations ranging from 2000 to 4000 mg/kg dry soil. Glucose was added as slurry with ASTM type I water and sprayed onto the soil using a spray bottle. The soil was then mixed well for 20 s. Triplicate samples of 50 g (dry soil basis) were weighed into separate SIC, attached to the respirometer, and incubated at 22°C. CO_2 measurements were made every 2 h. The optimal glucose concentration was determined to be 2500 mg/kg. This concentration was used for subsequent SIR assays.

SIR assays for 2,4-DNT were prepared, as described above for BR assays, by spraying a glucose water slurry to add 2500 mg/kg of glucose to the soil. Soil was then brought to about 55% of the SSL soil WHC with ASTM type I water, if necessary. The SIC were attached to the respirometer within an hour after glucose addition, and the rate of CO_2 evolution measurements were made every 96 min. SIR was measured on day 0 and day 28. An increase in CO_2 production was associated with new biomass production, and the rate of CO_2 measured prior to this event was used in the biomass carbon (Biomass C) calculation. The equation used to determine Biomass C was $X = 40.04y + 0.37$, where X = the total microbial Biomass C, and y = minimal initial rate of CO_2 evolution as (mL of CO_2 h^{-1} $100g^{-1}$ dry soil). Data collected on day 28 was used to estimate the EC values for the EM.

3.12.3. Soil enzyme activity assays

Dehydrogenase activity in moist soil was determined using 2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyltetrazoliumchloride (INT) as substrate (ISO 23753-2; 2005b). For each soil, 2.5 $\pm$ 0.1 g of moist soil was added to a glass vial with 2.5 mL of INT. The soil slurries were vortexed, and were incubated in the presence of INT for 24 $\pm$ 2 h at 37°C. At the end of the exposure period, ten mL of tetrahydrofuran (THF) were added to each vial. Controls received the THF before incubation at 37°C. Each vial was vortexed 1 min, and contents filtered using 0.45 µm filter cartridges and syringes. The first 2 mL of filtrate was discarded, and the remainder captured for analysis. When present in soil, the dehydrogenase enzyme induces the formation of formazan (INF). This colored compound is measured by spectrophotometry at 436 nm. The amount of formazan produced is measured against a standard curve of iodonitrotetrazolium formazan. A decrease in dehydrogenase activity is correlated to a decrease in formazan produced (Rossel et al. 1997; Gong et al., 1999a).

The potential activities for P-acquiring or N-acquiring enzymes were assessed using the method described by Sinsabaugh et al. (1997) and were based on ISO 14238, 15685, and 22939 methods (Table 96). Briefly, a slurry was prepared using 1 g of amended soil and 100 mL acetate buffer (pH 5.0). The slurry was vortexed for 1 min, and mixed for 15 min using a rotary mixer. Under continuous shaking, 200 µL slurry were taken and added into a 96-well black microplate. Separate microplates were prepared for different enzyme activities tested. Standard solutions of 4-methylumbelliferyl (MUB) phosphate for acid phosphatase or 4-MUB-N-acetyl-beta-glucosaminide for beta-N-acetyl glucosaminidase were prepared in sterile ASTM type I water. Fifty µL of the corresponding standard solution was added to each microplate well. Each microplate was incubated at room temperature (20-22°C). Assays were terminated by addition of ten µL of 0.5 N NaOH to each well of the microplate. Fluorescence was determined after 15 min for acid phosphatase and 40 min for N-acetyl glucosaminidase using a Bio-Tek Synergy HT-I fluorimeter set at 360-nm excitation and a 460-nm emission. Enzyme activity (expressed as nmol/h/g dry soil) was calculated as the rate of accumulation of product equivalents.

3.13. Bioaccumulation assessment

3.13.1. Effect of soil hydration on the uptake of RDX by earthworms

The effect of soil hydration on RDX uptake by earthworms was studied according to the American Society for Testing and Materials (ASTM) standard guide (ASTM, 1998). Earthworms (*E. andrei*) were acclimated for 24 h in non-amended TSL soil prior to the experiment. TSL soil was selected because it supports high bioavailability of RDX and could reveal any difference in uptake at this low RDX concentration (10 mg/kg) before such difference could be revealed in other soil types. Individual groups of six clitellated earthworms weighing from 300 to 600 mg were exposed to 60 g d. w. of amended soils, in triplicate. A time series study of RDX uptake by the earthworms in TSL soil amended at 10 mg/kg was conducted using exposures of 1, 2, 7, 9, and 14 d at two levels of soil hydration (75 or 95% of the WHC). The interstitial water was extracted from soil at the beginning of the experiment, using the method described in the 2006 ER-1416 Annual Report (Savard et al., 2010). The soil moisture levels were confirmed. Samples of bulk soil, interstitial water, and tissue were extracted, and analyzed for RDX and its metabolites using the modified USEPA Method 8330A.

3.13.2. Determination of lipid content in earthworms

Lipids are the primary storage compartment for hydrophobic organic chemicals in tissue. Some EMs had preferential solubility to lipids. For example, RDX is among such organic chemicals and has a six-fold preferential solubility to lipids than to water, based on the log K_{ow} values that range from 0.81 to 0.87 (cited by Talmage *et al.*, 1999). Changes in the lipid content can be consequential for the accumulation of these lipid soluble chemicals in earthworms. Therefore, we conducted experiments to test the hypothesis that exposure to RDX in soil can alter the lipid content in earthworms and affect RDX accumulation in their tissue.

Earthworms (*E. andrei*) with well-developed clitellum and weighing from 300 to 600 mg were acclimated for 24 h in non-amended TSL soil, and then exposed in TSL amended with RDX

concentration of 10 mg/kg. The lipid content of the earthworm tissues was determined after 0, 7, and 14 d of exposure in soil using the method of de Boer as described in Egeler *et al*. (2005). Briefly, depurated earthworms were frozen at -20°C for at least 2 h, and thawed in an ice water-bath. Individual tissue samples ranging from 0.5 to 1.0 g were placed in 50-mL glass vessels, to which a mixture of 8 mL 2-propanol and 10 mL cyclohexane was added. Samples were homogenized for 2 min using a Polytron tissue homogenizer. Water was then added, and samples were homogenized for one min or until the formation of an emulsion. Concentrated HCl (37%) was added drop-wise using a Pasteur pipette, while gently stirring the mixture until the disappearance of the emulsion. The samples were centrifuged at 450 x g for 5 min to separate the two phases. The volume of the upper phase (cyclohexane) was quantified and the extract was stored. Ten mL of the propanol/cyclohexane mixture described above was added to the lower phase (propanol), and then centrifuged at 450 x g for 5 min. The upper phase (cyclohexane) was quantified and combined with the previous cyclohexane extract. The combined sample was concentrated to 3 mL under a dry nitrogen stream, and transferred to a 6-mL pre-weighed glass vessel. The sample was then completely evaporated using a nitrogen stream and the vessel was placed in an oven at 150°C for 1 h. After cooling, dry vessel was weighed, and the lipid content was calculated by differences between the empty vessel and vessel with lipid, and expressed as the percent of wet weight.

3.13.3. Uptake of non-labeled energetic materials (EMs) by the earthworms in amended soil

The EMs uptake experiments were performed with SSL soil using *E. andrei*, according to the ASTM standard guide for soil bioaccumulation studies (ASTM 2004). EM treatments ranging from 0 (control) to 10,000 mg/kg, which were selected to represent environmentally relevant concentrations at contaminated sites or sub-lethal concentrations (Talmage *et al*., 1999; Robidoux *et al*., 2000; Simini *et al*., 2003; Best *et al*., 2006). Earthworms were acclimated for 24 h in non-amended SSL soil prior to the experiment. Six earthworms with well-developed clitellum and weighing 300 to 600 mg were placed into each replicate test unit (250 mL glass jar) containing 60 g (dry wt) soil. Two g of dry cereal was added to each test unit. Each test unit was then covered by a perforated lid to control soil moisture. Earthworm wet weights were recorded at the start of the experiment.

Different moisture-equilibration periods were tested to determine the optimal design for the bioaccumulation test. In equilibration studies, different batches of soil were amended with EM (up to 100 mg/kg), and hydrated individually to 75% of the SSL water holding capacity (WHC; 21% water on the basis of the dry SSL soil mass). Moisten soil was left in dark for 1, 4, or 7 d. Earthworms were then exposed to these hydrated amended soil. At the end of the exposure period, the earthworms were collected, counted, rinsed with ASTM type I water, and depurated for 24 h on a moistened filter paper. The earthworms were then rinsed, blotted-dry, placed into glass tubes, and were immediately frozen at –80°C. Soil samples from each replicate container were homogenized and stored at –20°C until processed for HPLC analysis as describe above. Measurements of EMs in tissue and soil samples were taken before and after exposure of earthworms. Experiments were repeated with optimal moisture-equilibration period for the determination of bioaccumulation factor (BAF).

Studies were conducted in triplicates with EMs amended in SSL2007d soil, and uptake of EMs in earthworms was measured following 0.25, 1, 2, 3, 7, or 14 d exposure time for all EMs tested. Additional exposure times were 0.08 d for both TNT and 2,4-DNT, or 28 d for HMX. At least three sub-lethal concentrations of EM were tested for bioaccumulation. The nominal concentrations chosen were: 1, 10, 100 mg/kg for RDX; 1, 10, 100, 1000, 10000 mg/kg for HMX; 10, 50, 100 mg/kg for TNT; 10, 20, 50 mg/kg for 2,4-DNT; and 25, 50, 100, 150 mg/kg for NG. The concentrations of EM and respective metabolites (MNX for RDX; 2-ADNT and 4-ADNT for TNT; 2A-4NT for 2,4-DNT; DNG for NG) were monitored in soil and in the earthworms during the uptake phase. The transformation of EMs in soil without earthworms was also determined.

The BAF values for individual EM treatments were calculated as the ratio of EM concentration found in earthworm tissue and in soil at steady state, or at the end of the experiment. When needed, steady state determination was done using Analysis of Variance (ANOVA) of the tissue EM data with SYSTAT 7.01 (SPSS Inc., Chicago, IL). Fisher's Least Significant Differences (FLSD) pairwise comparison test was used for comparisons among treatments. A significance level of $p \leq 0.05$ was accepted for all statistical analyses.

3.13.4. Effect of the earthworm loading rate on ^{14}C-EM uptake by the earthworms

The OECD draft test guideline for conducting soil bioaccumulation tests (OECD, 2010) suggests the use of 3-5 mg of earthworm per gram of wet soil. This corresponds to only one worm for 68 g of wet SSL soil. Such low earthworm loading rate could be insufficient for quantifying some EM uptake by the earthworms due to the low specific activity of these ^{14}C-EM and the quantities of ^{14}C-activity recovered from the exposed earthworms. Our earlier studies indicated that at least six worms per 60 g dry soil were necessary for analyses of the extractable and non-extractable ^{14}C-activity in tissues. In the following studies, we examined the effect of the earthworm loading rate on EM accumulation in earthworms, using RDX as a model compound. Earthworms (*E. andrei*) with well-developed clitellum and weighing from 300 to 600 mg were prepared for testing as described above and were exposed to 100 mg ^{14}C-RDX/kg SSL soil (nominal concentration) for 7 d. The earthworm loading rates included 1, 6 or 10 earthworms per 60, or 100 g of dry soil. Each loading rate treatment was replicated (n=3). Soil and tissue residue concentrations were analyzed using the sample oxidizer, model 307 (Perkin Elmer LAS Canada, Woodbridge, ON).

3.13.5. Accumulation of selected ^{14}C-EMs in earthworms using the kinetic approach

A kinetic bioaccumulation test consisted of two phases as described by others (Bruns *et al*., 2001; Jager *et al*., 2005; Vijver *et al*., 2005; Egeler *et al*. 2005; OECD, 2010). The uptake (exposure) phase and the elimination (post-exposure) phase were done with ^{14}C-EM amended SSL soil at relevant concentration (100 mg/kg for RDX; 10 mg/kg for HMX, and 50, and 100 mg/kg for TNT) using the methods described in Sarrazin *et al*. (2009).

Earthworms were acclimated for 24 h in non-amended SSL soil prior to the experiment. Then, earthworms with well-developed clitellum and weighing from 300 to 600 mg were exposed to ^{14}C-EM amended soils using the earthworm bioaccumulation microcosm (EBM) described in Sarrazin *et al.* (2009). Each treatment concentration of chemical was replicated (n=3). The uptake kinetics were quantified by a time-series sampling until a plateau or a steady state of the

EM concentration in the earthworms is reached. Uptake of radioactivity was assessed after 0.25 to 14 d. Then the earthworms exposed to ^{14}C-EM for up to 14 d were removed from soil, rinsed with ASTM Type I water, and transferred (without depuration) to a non-amended SSL soil. An elimination phase was necessary to determine the rate at which the ^{14}C-EM was excreted by the earthworms. Elimination of radioactivity was assessed after 0.08, to 14 d. The residual EM concentration in the earthworms at the end of the elimination phase was determined as a secondary endpoint. The concentration of ^{14}C-EM and its ^{14}C-metabolites in the earthworms was monitored during the uptake and elimination phases of this bioaccumulation test.

Based on the non-labeled experiments results, soil was separately amended with ^{14}C-RDX at 10 and 100 mg/kg, ^{14}C-TNT at 50, and 100 mg/kg, and with ^{14}C-HMX at 10, and 100 mg/kg. Earthworms were added to individual jars containing the ^{14}C-EM amended or the control soil, and each replicate jar was placed randomly in a desiccator unit. Uptake of ^{14}C-compound was determined by measuring radioactivity in tissue and soil samples at different exposure time. In order to determine a kinetic-based bioaccumulation factor (BAF_K) in earthworms, an elimination phase was added where the ^{14}C-EM exposed earthworms were transferred to an EM-free SSL soil for depuration. Radioactivity was determined in tissue and soil samples after 0.25, 1, 2, 7, and 14 d of elimination phase for ^{14}C-RDX; 0.25, 7, and 15 d of elimination phase for ^{14}C-TNT, and 1, 2, 7, 14, and 21 d for ^{14}C-HMX.

Data generated are further analyzed in order to determine the kinetic bioaccumulation factor (BAF_K). A toxicokinetic model for bioaccumulation is applicable when the parent compound remained in the soil and is not transformed during the study (Bruns et al. 2001). This was the case for both RDX and HMX. However, TNT, 2,4-DNT, and NG disappeared in soil during the experiment in absence of earthworms as described in Results (section 4.6 of this report).

When a kinetic approach is applicable, the BAF_K was calculated as the ratio of the rate constant (k_1) for EM uptake from soil (uptake of total radioactivity) and the rate constant (k_2) for EM elimination from the earthworms (elimination of extractable radioactivity). The goodness of fit of the models was determined using the coefficients of determination. The uptake of ^{14}C-compounds in earthworms for different periods of exposure to ^{14}C-EM in SSL soil was estimated using the one compartment exponential model for first-order kinetics modified from Bruns *et al*. (2001):

$$[\mathrm{EM_T}]_{\mathrm{total}} = \frac{k_1}{k_2} \times [EM_S] \times (1 - e^{-k_2 t}) \qquad (1)$$

where $[\mathrm{EM_T}]_{\mathrm{total}}$ is the total radioactivity (dpm/g dry wt tissue) in the earthworm tissue (^{14}C in extractable plus non-extractable fractions), k_1 is the uptake rate constant (g dry wt soil/g dry wt tissue/d), k_2 is the elimination rate constant (per d), $[\mathrm{EM_S}]$ is the concentration of radioactivity in dry soil (dpm/g dry wt soil) at the end of EM exposure (14 d) , and t is the duration of uptake (in days).

The elimination of extractable ^{14}C-labelled compounds from the earthworms was estimated using the exponential model modified from Bruns *et al*. (2001):

$$[EM_T] = ([EM_T]_{SS} \times e^{-k_2 t}) + [EM_T]_R, \quad (2)$$

where $[EM_T]$ is the radioactivity (dpm/g dry tissue) in the tissue, $[EM_T]_{SS}$ is the radioactivity in the earthworm (dpm/g dry tissue) at an apparent steady-state prior to start of elimination phase, k_2 is the elimination rate constant (d^{-1}), $[EM_T]_R$ is the residual radioactivity (dpm/g dry tissue) at the end of elimination phase, and t is the duration of elimination phase (in days). The coefficient of determination (R^2) was estimated for each fitted curve. Nonlinear regression models were run in SYSTAT 7.01 (SPSS, Chicago, IL) and KaleidaGraph version 4.03 (Synergy Software, Reading, PA) for the iterative curve-fitting procedures. Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Data were not transformed or weighted.

These models fit the accumulation data and estimate the uptake (k_1) and elimination (k_2) rate constants. The BAF_K was calculated as the ratio of uptake to elimination rate constants (k_1/k_2) as described below.

Under steady state conditions, $[EM_T]_{SS} = \frac{k_1}{k_2} [EM_S]_{SS}$ (3)

And the $BAF_K = \frac{k_1}{k_2}$ (4)

where $[EM_S]_{SS}$ is the radioactivity in the soil (dpm/g dry wt soil) at apparent steady-state conditions, the BAF_K (expressed as g dry wt soil/g dry wt tissue) is the ratio of the tissue uptake rate constant (k_1) to the tissue elimination rate constant (k_2).

As part of the mass balance studies, we also examined the ^{14}C-activity in the acetonitrile-extractable as well as the non-extractable fractions to follow the fate of absorbed ^{14}C-EM in the earthworm. The radioactivity in the acetonitrile extractable, and the non-extractable fractions of the earthworm tissues was analyzed using liquid scintillation counting (LSC), and was expressed as disintegrations per minute (dpm). Radioactivity in the extractable fraction was considered to represent EM or its unbound degradation products, whereas radioactivity in the non-extractable fraction was considered to represent EM degradation products that were bound to cellular constituents.

3.13.6. Evaluation of the plant microcosm units for assessing the EM accumulation in ryegrass

EMs uptake by plants was determined in SSL soil according to standard protocols used for toxicity assays (ASTM, 2002; USEPA, 1996). Twenty seeds of ryegrass *Lolium perenne* were sown in 10-cm wide pots each containing 200 g of dry soil. ASTM type I water was added to obtain 75% of the WHC of SSL soil. A sub-lethal soil RDX concentration (30 mg/kg) was chosen for exposure based on the results of our previous studies. Three sets of plant pots were prepared, and included both controls and RDX-amended samples in triplicate; the first set contains unlabeled material, and was placed in a 1-L polyethylene bags closed with an elastic

band to minimize loss of soil water as done for the phytotoxicity studies. The second set also contains unlabeled material, and was placed in the plant accumulation microcosms (PAM) which were constructed from clear polycarbonate vacuum desiccators (23 cm inner diameter). These units were tightly closed, and also permit to avoid loss of soil water due to evapo-transpiration. All plant pots were incubated in the dark for the first two days and then exposed to a diurnal cycle of 16 h of light (5000±500 lux) and 8 h in the dark for 21 or 34 days. The third set, contains ^{14}C-RDX. Plants exposed to non-labeled material in microcosms were aerated once a week.

A beaker with 20 mL of 0.5 N KOH was placed inside the PAMs which contain ^{14}C-labeled material. The collection of KOH in this internal alkali trap was done three times a week using an access hole (3 mm) drilled in the top of the microcosm. The external port was connected as an outlet to a series of four test tubes. The first tube contained water and acted as an anti-vacuum trap. The other tubes contained each 20 mL of 0.5 M KOH to trap evolved CO_2. Air was collected 3-times each week using a pump that was connected to the third trap. Total air flush of the microcosm was done within 4 h. All traps were sampled (1 mL) and mixed with ASTM Type I water (1 mL) after each air flush. Scintillation counting fluid was added (18 mL) and radioactivity in these samples was counted in a Packard Tri-Carb 2100 TR liquid scintillation counter. Plants remained healthy in the PAM for 34 days, after which their leaves started to change color.

All plant pots were aerated once a week. Both shoots and roots were harvested at the end of the exposure period. The soil was washed away from roots with ASTM type I water. Excess water was absorbed with a paper towel. Shoots and roots were kept at -20°C prior to lyophilization and explosive extraction.

Lyophilized plant tissues (shoots or roots) were ground using mortars and pestles, then transferred to a glass conical tube and 500 µl of internal standard solution was added to each tube, and vortexed 1 min. All internal standards were prepared in acetonitrile to reach a maximum concentration of 1 mg/L. All samples were sonicated (Branson Model 3200, Danbury, CT) for 18 ± 2 h at 20°C, and centrifuged 1500 rpm for 1 h. Supernatant (between 0.3 and 0.4 mL) was mixed with an equivalent amount of calcium chloride solution (5 g/L) and kept at 4°C for 24 h. Supernatants were then filtered through a 0.45 µm filter and analyzed by HPLC using a modification of the USEPA method 8330.

3.13.7. Uptake of non-labeled EMs by ryegrass in amended soil

The following EM were tested: RDX, HMX, TNT, 2,4-DNT, and NG. At least three sub-lethal concentrations of EM were tested for the study. The choice of concentrations was based on toxicity results. Plants were exposed to the EM-amended soil and harvest after 14, 21, 28, and 34 d of growth in the PAM. All treatments were carried out in triplicates. Analyses of shoots and roots tissues were performed as described above.

For non-labeled studies, between 0.003 and 0.080 g of tissue was mixed with 0.5 mL of acetonitrile containing the appropriate internal standard (e.g. HMX for RDX; RDX for HMX). Plant extracts were sonicated in the dark at 20 degrees C for 18 h. Extracts were centrifuged at

1500 rpm for 1 h. At each time period (14, 21, 28, and 35 d), bioconcentration factors (BCF) were calculated by dividing the concentration of EM in tissue by the measured total concentration of EM in the soil. The BCF values were determined at 10, 30, and 100 mg/kg nominal soil concentrations.

3.13.8. Uptake of ^{14}C-EM by ryegrass in amended soil

The same methodology was used for both ^{14}C-EM amended soil and unlabeled experiments. Radioactive material was available only for RDX, HMX, and TNT. The plant accumulation microcosm was made air tight and had one internal and three external vials with 0.5 N KOH used to collect the evolved $^{14}CO_2$ within the PAM. A 3-mm access port on the top of each PAM allowed the sampling of the internal PAM alkali trap for CO_2. An anti-vacuum trap filled with water was placed in series with the three external CO_2 traps. The last external CO_2 trap was connected to a tube which contained an outlet to flush air three times each week using a vacuum pump (Figure 2). Total air flush of the PAM was completed within 4 h based on preliminary studies.

On the designated sampling day, each external trap was sampled (1 mL) and mixed with ASTM Type I water (1 mL) after each air flush. A 1-mL aliquot was also taken from each internal CO_2 trap. Scintillation counting fluid (18 mL) was added to the samples, and radioactivity was determined using a Packard Tri-Carb 2100TR (Canberra, Concord, ON, Canada) liquid scintillation counter.

For non-labeled studies, both shoots and roots were harvested at the end of exposure period 14, 21, 28, and 34 d. The soil was washed away from roots with ASTM type I water. Excess water was absorbed with a paper towel. Shoots and roots were kept at -20°C prior to lyophilization. From 0.02 to 0.7 g dry tissues were combusted using an Oxidizer (model 307, Perkin Elmer).

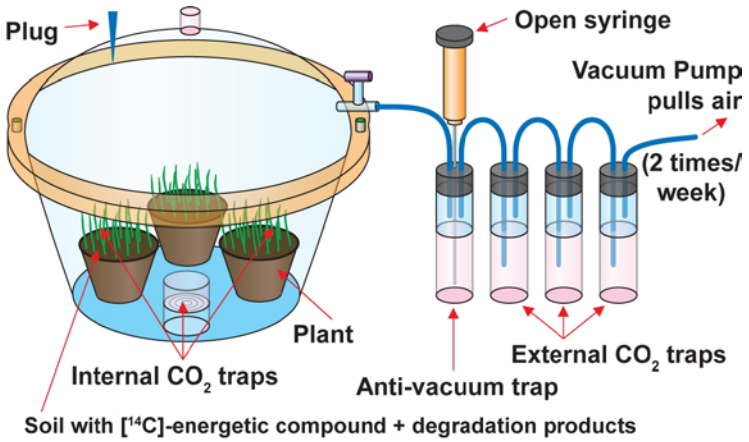


Figure 2. Schematic model of the plant accumulation microcosm used for ^{14}C-labeled EM studies.

3.14. Data analyses

Ecotoxicological data were analyzed using regression models selected among those described in Environment Canada Guidance Document (EC, 2005). During the model selection process, compliance with the normality assumptions and homoscedasticity of the residuals were determined by examining the stem-and-leaf graphs and histograms of the residuals. The best fit was evident when the regression lines generated by the models were closest to the data points, the regression coefficients for point estimates were the greatest, the residuals were homoscedastic (i.e., had most random scattering), and the means, standard errors, and variances of the residuals were the smallest. The models selected for data analyses in these studies included logistic (Gompertz) [1], logistic hormetic [2], exponential [3], and linear [4].

$$Y = a \times e^{\{[\log(1-p)]\times(C\div ECp)^b\}} \quad [1]$$

$$Y = \frac{a\times[1+(h\times C)]}{1+[(p+(h\times C))\div(1-p)]\times[C\div ECp]^b} \quad [2]$$

$$Y = a \times e^{(([\log(1\text{-}p)] \div ECp) \times C) + b} \quad [3]$$

$$Y = [(-a \times p) \div ECp] \times C + a \quad [4]$$

where Y = dependent variable for a measurement endpoint (e.g., number of juveniles or shoot mass); a = the y-axis intercept (i.e., the control response); e = the exponent of the base of the natural logarithm; p = desired value for 'p' effect (e.g., 0.50 for a 50% decrease from the control response; EC_{50}); C = the exposure concentration in test soil; ECp = estimate of effect concentration for a specified percent effect; h = the hormetic effect parameter; and b = a scale parameter that defines the shape of the equation. Data that exhibited hormesis, a concentration-response phenomenon characterized by a low-dose stimulation and high-dose inhibition (Calabrese, 2008), were fitted to the hormetic model. The ECp parameters used in these studies included the EM concentration producing a 20% (EC_{20}) or 50% (EC_{50}) decrease in the measurement endpoint compared with carrier control. The EC_{20} parameter based on reproduction (soil invertebrates) or growth (terrestrial plants) endpoints is the preferred parameter for deriving Eco-SSL values. The EC_{50} parameter, a commonly reported value, was included to enable comparisons of the results produced in these studies with results reported by other researchers. The 95% confidence intervals (CI) associated with the point estimates were determined.

Analysis of Variance (ANOVA) or Analysis of Covariance (ANCOVA) was used to determine the bounded (when possible) no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) values for survival, reproduction, growth, and biological activity data. When no-observed-adverse-effect-concentration (NOAEC) or lowest-observed-adverse-effect-concentration (LOAEC) values were determined, the same statistical methods were used. Mean separations were determined using Fisher's Least Significant Difference (FLSD) pairwise comparison test. Student's *t*-test (two-tailed) was used for pairwise comparisons. The

relationships among the selected soil parameters and toxicity data were determined using Pearson's correlation analysis. All analyses, except estimation of annual decomposition rate constants (k) (see Section 3.12.1), were done using untransformed data and analytically determined EM concentrations. A significance level of $p \leq 0.05$ (95% confidence level) was accepted for all statistical tests. Statistical analyses were performed using SYSTAT® 11 (Systat Software, Inc., Chicago, IL, USA) or TOXCALC (Tidepool Scientific Software).

4. Properties of test soils

Several batches of SSL soil were used throughout the SERDP-funded projects. In order to obtain SSL soil having the required soil properties, multiple sub-samples were collected from different field sites in the coastal plain on the property of APG. Sub-samples were homogenized, sieved, and mixed to prepare a composite sample from each of the field sites. The composite samples were analyzed for physical texture, basic micro- and macro-nutrients, pH, cation exchange capacity, and organic matter content to determine which site had the key soil properties that qualify as SSL soil. Analytical results were used to identify the best field site for collecting a sufficient quantity of SSL soil to perform the remaining project tasks. The selected soil was sieved (5 mm) and dried at room temperature (20-25°C). The SSL2000 soil batch was used for toxicity testing during SERDP CU-1221 project (Table 1). The SSL2004 and SSL2007d soil batches were used for toxicity testing in the present SERDP ER1416 project. Table 2 indicates which batch of SSL soil was used for each toxicity test. The SSL2007e and SSL2011 batches were collected from the same location in the coastal plain of APG for use in the microbial and litter decomposition studies. These studies were designed to assess the effects of energetic materials on the biological activity endpoints in test soil, and thus required the use of freshly-collected soil with a level of activity that was representative of the field conditions. Replicate sub-samples of this soil were also analyzed and the results confirmed the similarity between the SSL2007d soil and the SSL2007e soil batch. Additional chemical characteristics of the SSL2007d, SSL2007e and SSL2011 soil batches are presented in Table 3.

The KL2006 batch was used in the definitive soil invertebrate toxicity testing of 2,4-DNT and in initial phytotoxicity testing of 2,4-DNT. The KCL2002 batch was used in the repeat phytotoxicity testing of 2,4-DNT using alfalfa. The TSL2002 batch soil was used in the definitive soil invertebrate and plants toxicity testing of 2,4-DNT and HMX.

Table 1. Selected physico-chemical characteristics of Teller sandy loam (TSL), Sassafras sandy loam (SSL), Kirkland clay loam (KCL), Kirkland loam (KL), and Webster clay loam (WCL) soils.

Soil property	SSL 2000	SSL 2004	SSL 2007d	SSL 2007e	SSL 2011	TSL 2002	KCL 2002	KL 2006	WCL 2001
pH	5.2	4.4	4.9	5.0	4.8	4.4	6.4	5.7	5.9
Organic matter (%)	1.2	2.0	2.3	2.2	0.8	1.4	2.6	1.5	5.3
Sand (%)	71	70	55	62	68	65	37	39	33
Silt (%)	13	16	28	25	23	22	34	42	39
Clay (%)	17	14	17	13	9.5	13	29	19	28
CEC (cmol/kg)	5.5	9.6	9.3	7.8	6.0	4.3	10	13	21
WHC (%)	18	18	25	25	18	13	20	20	23

Table 2. Sassafras sandy loam soil batches used for toxicity testing.

SSL2000	SSL2004	SSL2007d	SSL2011
2,4-DNT definitive soil invertebrate toxicity tests	Positive control (boric acid) in 2005	Positive control (boric acid) in 2007	Soil respiration studies
	Positive control (boric acid) in 2006	2-ADNT definitive soil invertebrate and terrestrial plant toxicity tests	
	2,4-DNT definitive terrestrial plant toxicity tests	4-ADNT definitive soil invertebrate and terrestrial plant toxicity tests	
		NG definitive soil invertebrate and plant terrestrial toxicity tests	

Table 3. Additional chemical characteristics of Sassafras sandy loam soil collected in 2007.

Soil Parameter	SSL2007d	SSL2007e	SSL2011
Conductivity mmhos/cm	0.07	0.08	0.03
Total C %	nd	1.3	nd
Total N %	nd	0.11	nd
N (NO_3) mg/kg	nd	1.5	0.6
N (NH_4) mg/kg	nd	1.4	3.8
Ca mg/kg	335	270	69
Mg mg/kg	87	73	30
P mg/kg	6	5	8
S mg/kg	24	15	7.4
K mg/kg	74	75	31
Cu mg/kg	9	24	4.9
Zn mg/kg	11	18	2.6

Table note: nd = parameter was not determined.

4.1. Analytical determinations of 2,4-DNT in soil treatments used in definitive toxicity tests with soil invertebrates and terrestrial plants

Weathering-and-aging of 2,4-DNT was performed in TSL (TSL2002), KCL (KCL2002), KL (KL2006), and WCL (WCL2001) at ECBC as single batches for toxicity studies. These soils were selected for their physical and chemical characteristics that support different levels of 2,4-DNT bioavailability. Analytically determined concentrations of 2,4-DNT in each soil are presented in Tables 4 to 8. Percentage recovery of 2,4-DNT ranged between 52 and 84 in the TSL2002 soil, between 41 and 65 in the KCL2002 soil, between 56 and 81 in the KL2006 soil, and between 52 and 72 in the WCL2001 soil.

Table 4. Nominal and analytically determined concentrations of 2,4-DNT at the beginning (initial) and at the end of weathering-and-aging in Teller sandy loam (TSL2002) soil treatments used in the definitive plant toxicity tests.

Nominal concentration (mg/kg)	Analytically determined concentration before weathering-and-aging (mg/kg)	Analytically determined concentration after weathering-and-aging (mg/kg)	Recovery after weathering-and-aging (%)
Negative control	BDL	BDL	NA
Carrier control	BDL	BDL	NA
2	3.1 ± 0.1	1.6 ± 0.04	52
5	5.2 ± 0.1	3.1 ± 0.1	60
10	10 ± 0.3	6.1 ± 0.04	60
15	15 ± 0.2	10 ± 0.3	67
20	22 ± 1	15 ± 1	66
40	42 ± 7	26 ± 1	61
60	59 ± 6	43 ± 1	73
80	83 ± 2	63 ± 1	76
160	163 ± 4	118 ± 4	72
300	293 ± 5	246 ± 15	84

Table notes: BDL= below analytical detection limit (0.1 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

Table 5. Nominal and analytically determined concentrations of 2,4-DNT at the beginning (initial) and at the end of weathering-and-aging in Teller sandy loam (TSL2002) soil treatments used in the definitive soil invertebrate toxicity tests.

Nominal concentration (mg/kg)	Analytically determined concentration before weathering-and-aging (mg/kg)	Analytically determined concentration after weathering-and-aging (mg/kg)	Recovery after weathering-and-aging (%)
Negative control	BDL	BDL	NA
Carrier control	BDL	0.4 ± 0.1	NA
2	2.3 ± 0.02	1.7 ± 0.1	75
5	5.3 ± 0.1	3.7 ± 0.1	70
10	11 ± 0.1	7.5 ± 0.3	72
15	15 ± 0.5	11 ± 0.5	71
20	21 ± 0.3	15 ± 0.4	69
40	42 ± 1	29 ± 1	69
60	64 ± 0.3	44 ± 1	69
80	84 ± 3	63 ± 2	76
160	170 ± 3	127 ± 1	74

Table notes: BDL= below analytical detection limit (0.1 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

Table 6. Nominal and analytically determined concentrations of 2,4-DNT at the beginning and end of weathering-and-aging in Kirkland clay loam (KCL2002) soil treatments used in the definitive toxicity tests with barnyard grass and perennial ryegrass.

Nominal concentration	Analytically determined concentration before weathering-and-aging (mg/kg)	Analytically determined concentration after weathering-and-aging (mg/kg)	Recovery after weathering-and-aging (%)
Negative control	BDL	BDL	NA
Carrier control	BDL	BDL	NA
2	2.0 ± 0.0	0.80 ± 0.02	41
5	5.6 ± 0.1	2.5 ± 0.1	45
10	11 ± 0.2	4.6 ± 0.1	43
15	15 ± 0.4	6.6 ± 0.1	44
20	20 ± 0.3	8.7 ± 0.1	43
40	42 ± 0.3	18 ± 0.4	42
60	63 ± 1	29 ± 0.5	46
80	82 ± 1	36 ± 13	44
100	110 ± 10	51 ± 2	47
120	127 ± 4	63 ± 2	50
160	160 ± 2	87 ± 2	54
200	212 ± 2	115 ± 1	54
250	248 ± 3	157 ± 1	63
300	304 ± 6	175 ± 3	58
400	391 ± 0.2	239 ± 6	61
600	632 ± 12	408 ± 30	65

Table notes: BDL= below analytical detection limit (0.1 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

Table 7. Nominal and analytically determined concentrations of 2,4-DNT at the beginning (initial) and at the end of weathering-and-aging in Kirkland loam (KL2006) soil treatments used in the definitive soil invertebrate toxicity tests and in toxicity test with alfalfa.

Nominal concentration (mg/kg)	Analytically determined concentration before weathering-and-aging (mg/kg)			Analytically determined concentration after weathering-and-aging (mg/kg)			Recovery after weathering-and-aging (%)
Negative control	BDL			BDL			NA
Carrier control	BDL			BDL			NA
2	2.7	±	0.1	1.77	±	0.03	66
5	5.5	±	1.0	3.6	±	0.3	64
10	10	±	1	7.1	±	0.1	70
20	21	±	1	14	±	0.1	64
40	42	±	2	28	±	1	67
80	82	±	13	56	±	1	68
160	156	±	11	88	±	2	56
300	303	±	7	247	±	8	81
600	631	±	55	444	±	13	70

Table notes: BDL= below analytical detection limit (0.1 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

Table 8. Nominal and analytically determined concentrations of 2,4-DNT at the beginning and end of weathering-and-aging in Webster clay loam (WCL2001) soil treatments used in the definitive soil invertebrate and plant toxicity tests.

Nominal concentration	Analytically determined concentration before weathering-and-aging (mg/kg)			Analytically determined concentration after weathering-and-aging (mg/kg)			Recovery after weathering-and-aging (%)
Negative control	BDL			BDL			NA
Carrier control	BDL			BDL			NA
2	2.4	±	0.1	1.8	±	0.04	72
5	5	±	1	3.8	±	0.1	70
10	9.8	±	0.8	7.5	±	0.2	76
15	16	±	0.8	11	±	0.1	69
20	21	±	2	14	±	0.2	70
40	40	±	1	28	±	0.4	70
60	64	±	2	39	±	0.2	61
80	80	±	5	54	±	2	68
100	109	±	3	65	±	1	60
160	175	±	7	97	±	2	56
200	219	±	11	115	±	4	52
250	262	±	7	158	±	5	60
300	339	±	9	189	±	5	56
400	440	±	26	260	±	11	59
600	677	±	19	447	±	4	66

Table notes: BDL= below analytical detection limit (0.1 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.2. Analytical determinations of 2,4-DNT in SSL soil during the litter decomposition study

Concentrations of 2,4-DNT were analytically determined in each freshly amended treatment of SSL2007e soil to establish a baseline of initial concentrations for litter decomposition study. The initial concentrations of 2,4-DNT in the two lowest treatments were only 38% and 62% of nominal 10 and 100 mg/kg treatments, respectively. These comparatively low recovery rates suggest rapid biotransformation processes in freshly amended soils during the short (2-3 days) period in transit to analytical lab. The initial analytically determined 2,4-DNT concentrations were more consistent with the respective target concentrations in the greater nominal treatments of 1000 and 10000 mg/kg (Table 9). Concentrations of 2,4-DNT in each treatment were analytically determined on each straw harvest date during the eight-month study. Percent recovery declined steadily over time in 10, 100, and 1000 mg/kg nominal treatments, but at a greater rate in the lowest two nominal treatments (Table 9). Concentrations of 2,4-DNT remained relatively stable in the greatest nominal treatment of 10000 mg/kg, providing an indirect evidence for inhibition of microbial activity at this concentration.

Table 9. Nominal and analytically determined concentrations of 2,4-DNT in Sassafras sandy loam (SSL2007e) soil during the eight-month litter decomposition study with *Dactylis glomerata*.

Nominal concentration	10		100		1000		10000	
	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %
Measured Initial	3.8 (0.4)	38[†]	62 (3.2)	62[†]	1274 (19)	127[†]	9343 (501)	93[†]
1 Month	2.1 (0.02)	55	34 (1.6)	56	887 (14)	70	13467 (676)	144
2 Months	2.4 (0.4)	63	24 (0.8)	40	1024 (49)	80	12637 (569)	135
3 Months	1.1 (0.04)	30	16 (1.1)	27	843 (5)	66	10145 (899)	109
4 Months	1.4 (0.1)	37	12 (0.2)	20	853 (35)	67	12585 (1216)	135
6 Months	0.6 (0.1)	16	7 (0.4)	12	614 (54)	48	10893 (625)	117
8 Months	0.6 (0.01)	16	6 (0.6)	9	740 (73)	58	10958 (637)	117

Table notes: [†]Percent initial recovery of 2,4-DNT from freshly amended SSL soil compared with nominal target concentration; The remaining recovery values show percent change from the initial analytically determined concentration in freshly amended soil during the 8-month study. Measured concentrations are based on USEPA Method 8330A; Values are means (n=3) and Standard Deviations (SD).

4.3. Analytical determinations of 2-ADNT weathered-and-aged in SSL soil treatments used in range-finding toxicity tests

Results of analytical determination of 2-ADNT at the beginning and at the end of the 3-months weathering-and-aging process are presented in Table 10. No 2-ADNT was detected in the negative or carrier controls. The 47, 55-72, 57, and 65% decreases from the initial 2-ADNT concentrations in freshly amended soil were determined in the nominal treatments of 10, 50, 100, and 200 mg/kg, respectively, after the three-month weathering-and-aging of 2-ADNT in soil. Concentration of 2-ADNT remained relatively stable during weathering-and-aging in soil in the 400 mg/kg and greater nominal treatments of SSL2007d soil, with recovery rates $\geq$82 percent.

Table 10. Nominal and analytically determined concentrations of 2-ADNT at the beginning and at the end of weathering-and-aging in Sassafras sandy loam (SSL2007d) soil treatments used in the range-finding toxicity tests.

Nominal concentration (mg/kg)	Analytically determined concentration before weathering-and-aging (mg/kg)			Analytically determined concentration after weathering-and-aging (mg/kg)			Recovery after weathering-and-aging (%)
Plant toxicity tests							
Negative control	BDL			BDL			NA
Carrier control	BDL			BDL			NA
10	8.9	±	1.2	4.2	±	0.2	47
50	40	±	3	29	±	8	72
200	173	±	4	105	±	12	61
600	523	±	63	492	±	80	94
2000	1835	±	275	1742	±	117	95
Invertebrate toxicity tests							
Carrier control (acetone)	BDL			BDL			NA
50	45	±	4	25	±	1	55
100	89	±	2	51	±	2	57
200	187	±	12	121	±	10	65
400	350	±	19	293	±	8	84
800	813	±	102	670	±	36	82

Table notes: BDL= below analytical detection limit (0.05 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.4. Analytical determinations of 2-ADNT weathered-and-aged in SSL soil treatments used in definitive toxicity tests with soil invertebrates and terrestrial plants

Analytically determined concentrations of 2-ADNT in soil treatments are shown in Table 11. The recoveries of 2-ADNT ranged from 61 to 137 percent after the three-month weathering-and-aging in soil.

Table 11. Nominal and analytically determined concentrations of 2-ADNT at the beginning and end of weathering-and-aging in Sassafras sandy loam (SSL2007d) soil treatments used in the definitive soil invertebrate and plant toxicity tests.

Nominal concentration	Analytically determined concentration before weathering-and-aging (mg/kg)	Analytically determined concentration after weathering-and-aging (mg/kg)	% recovery after weathering-and-aging
Negative control	BDL	BDL	NA
Carrier control	BDL	BDL	NA
20	16 ± 0.4	10 ± 2	60
40	35 ± 2	21 ± 4	61
60	53 ± 4	34 ± 1	63
80	69 ± 3	45 ± 2	65
100	90 ± 4	58 ± 7	64
120	105 ± 2	76 ± 3	72
140	131 ± 5	90 ± 4	69
160	142 ± 3	99 ± 2	70
180	166 ± 2	120 ± 1	72
200	176 ± 3	129 ± 5	73
300	272 ± 3	241 ± 7	89
400	359 ± 10	314 ± 8	87
1000	884 ± 61	852 ± 1	96
2000	1170 ± 29	1597 ± 3	137
5000	4409 ± 218	4027 ± 10	91
10000	8937 ± 472	8287 ± 6	93

Table notes: BDL= below analytical detection limit (0.05 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.5. Analytical determinations of 2-ADNT freshly amended into SSL soil treatments used in definitive plant toxicity tests

The evaluation of the effects of 2-ADNT freshly amended into SSL soil on terrestrial plants was added to the project to test the hypothesis that weathering-and-aging 2-ADNT in SSL soil can affect its toxicity to terrestrial plants. This hypothesis was tested by comparing the phytotoxicity of 2-ADNT freshly amended into soil with that of 2-ADNT weathered-and-aged in soil presented in Section 4.4.4.6. Definitive plant toxicity tests were conducted in triplicate with 2-ADNT freshly amended into SSL2007d soil. Nominal concentrations selected for these tests were 0 (negative control), 0’ (carrier control), 100, 300, 1000, 3000, and 5000 mg/kg. Corresponding analytically determined concentrations of 2-ADNT in SSL2007d soil are presented in Table 12. Analytically determined 2-ADNT concentrations were comparable to nominal concentrations, with recovery ranging from 91 to 101%.

Table 12. Nominal and analytically determined concentrations of 2-ADNT freshly amended into Sassafras sandy loam (SSL2007d) soil treatments used in the range-finding toxicity tests.

Nominal concentration (mg/kg)	Measured concentration (mg/kg)	Recovery (%)
Negative control (H_2O)	BDL	NA
Carrier control (acetone)	BDL	NA
100	91 ± 1.8	91
300	286 ± 23	95
1000	991 ± 46	99
3000	3028 ± 128	101
5000	4929 ± 325	99

Table notes: BDL= below analytical detection limit (0.05 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.6. Analytical determinations of 2-ADNT in SSL soil during the litter decomposition study

Concentrations of 2-ADNT were analytically determined in each freshly amended treatment of SSL2007e soil to establish a baseline of initial concentrations for litter decomposition study. The initial analytically determined concentrations of 2-ADNT were consistent with the respective target concentrations (Table 13). Concentrations of 2-ADNT in each treatment were analytically determined on each straw harvest date during the eight-month study. Percent recovery declined steadily over time in 10, 100, and 1000 mg/kg nominal treatments, but at a greater rate in the lowest two nominal treatments (Table 13). Concentrations of 2-ADNT remained relatively stable in the greatest nominal treatment of 10000 mg/kg, providing an indirect evidence for inhibition of microbial activity at this concentration.

Table 13. Nominal and analytically determined concentrations of 2-ADNT in Sassafras sandy loam (SSL2007e) soil during the eight-month litter decomposition study with *Dactylis glomerata*.

Nominal concentration	10		100		1000		10000	
	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %
Measured Initial	10 (0.4)	100[†]	117 (5)	117[†]	1200 (55)	120[†]	10000 (500)	100[†]
1 Month	4.9 (0.04)	47	60 (4)	51	1297 (89)	109	8980 (423)	90
2 Months	4.34 (0.2)	41	51 (4)	44	1113 (107)	94	10142 (925)	101
3 Months	2.8 (0.2)	27	46 (3)	39	1079 (63)	91	10571 (517)	106
4 Months	2.1 (0.1)	20	33 (0.3)	28	804 (8)	68	8447 (40)	84
6 Months	1.4 (0.1)	13	22 (1)	18	938 (86)	79	8738 (213)	87
8 Months	1.2 (0.02)	12	18 (1)	15	1025 (26)	86	9815 (615)	98

Table notes: [†]Percent initial recovery of 2-ADNT from freshly amended SSL soil compared with nominal target concentration; The remaining recovery values show percent change from the initial analytically determined concentration in freshly amended soil during the 8-month study. Measured concentrations are based on USEPA Method 8330A; Values are means (n=3) and Standard Deviations (SD).

4.7. Analytical determinations of 4-ADNT weathered-and-aged in SSL soil treatments used in definitive toxicity tests with soil invertebrates and terrestrial plants

Analytical determinations of 4-ADNT from soil treatments after weathering-and-aging in SSL2007d established 4-ADNT exposure concentrations of 0, 0, 3, 8, 13, 12, 22, 28, 33, 59, 63, 64, 75, 150, 243, 727, 724, 3277, and 8634 mg/kg, respectively (Table 14). These results indicate that the recovery of 4-ADNT was between 13 and 74 percent after the three-month weathering-and-aging in soil.

Table 14. Nominal and analytically determined concentrations of 4-ADNT at the beginning and end of the weathering-and-aging process in Sassafras sandy loam (SSL2007d) soil.

Nominal concentration	Analytically determined concentration before weathering-and-aging (mg/kg)	Analytically determined concentration after weathering-and-aging (mg/kg)	Recovery after weathering-and-aging (%)
Negative control	BDL	BDL	NA
Carrier control	BDL	BDL	NA
20	23 ± 0.5	3 ± 0.3	13
40	48 ± 2	8 ± 0.1	18
60	69 ± 0.4	13 ± 1	19
70	87 ± 2	12 ± 1	14
80	100 ± 13	22 ± 2	22
100	118 ± 7	28 ± 2	24
120	153 ± 17	33 ± 2	22
140	173 ± 19	59 ± 5	34
160	204 ± 8	63 ± 8	31
180	226 ± 15	64 ± 6	28
200	275 ± 34	75 ± 3	27
300	404 ± 69	150 ± 19	37
400	590 ± 65	243 ± 12	41
1000	1217 ± 9	727 ± 17	60
2000	2621 ± 63	724 ± 22	28
5000	5964 ± 100	3277 ± 1260	55
10000	11655 ± 814	8634 ± 1253	74

Table notes: BDL= below analytical detection limit (0.05 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.8. Analytical determinations of 4-ADNT freshly amended into SSL soil treatments used in plant toxicity tests

In order to test the hypothesis that weathering-and-aging 4-ADNT in SSL soil can affect its toxicity to terrestrial plants, the phytotoxicity of 4-ADNT freshly amended into soil was measured. Nominal concentrations selected for these tests were 0 (negative control), 0’ (carrier control), 10, 20, 60, 180, 600, 2000, and 10,000 mg/kg. Corresponding analytically determined concentrations of 4-ADNT in SSL2007d soil are presented in Table 15. Analytically determined 4-ADNT concentrations were comparable to nominal concentrations, with recovery ranging from 66 to 87%.

Table 15. Nominal and analytically determined concentrations of 4-ADNT freshly amended into Sassafras sandy loam (SSL2007d) soil at the beginning of plant toxicity tests.

Nominal concentration (mg/kg)	Measured concentration (mg/kg)	Recovery (%)
Negative control (H_2O)	0.0	
Carrier control (acetone)	0.0	
10	6.6 ± 0.1	66
20	13 ± 0.2	66
60	45 ± 1	75
180	147 ± 5	82
600	516 ± 24	86
2000	1711 ± 147	86
10,000	8690 ± 365	87

4.9. Analytical determinations of 4-ADNT in SSL soil during the litter decomposition study

Concentrations of 4-ADNT were analytically determined in each freshly amended treatment of SSL2007e soil to establish a baseline of initial concentrations for litter decomposition study. The initial analytically determined concentrations of 4-ADNT were generally consistent with the respective target concentrations (Table 16). Concentrations of 4-ADNT in each treatment were analytically determined on each straw harvest date during the eight-month study. Percent recovery declined steadily over time in all nominal treatments, but at a greater rate in the lowest nominal treatment of 10 mg/kg, providing an indirect evidence for lower inhibition of microbial activity at this concentration compared with inhibition in greater nominal treatments (Table 16).

Table 16. Nominal and analytically determined concentrations of 4-ADNT in Sassafras sandy loam (SSL2007e) soil during the eight-month litter decomposition study with *Dactylis glomerata*.

Target concentration	100		1000		5000		10000	
	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %
Measured Initial	113 (9)	113[†]	1380 (34)	138[†]	5880 (561)	117[†]	12560 (606)	126[†]
1 Month	51 (0.4)	45	765 (73)	56	4216 (44)	72	7445 (451)	59
2 Months	45 (1)	40	1019 (3)	74	3903 (183)	66	7943 (1183)	63
3 Months	34 (0.3)	30	751 (58)	55	4049 (16)	69	8115 (98)	65
4 Months	29 (1)	26	703 (34)	51	3759 (332)	64	8133 (755)	65
6 Months	22 (1.5)	19	652 (53)	47	3909 (153)	66	7368 (238)	59
8 Months	14 (0.2)	12	675 (21)	49	3985 (107)	68	7700 (405)	61

Table notes: [†]Percent initial recovery of 4-ADNT from freshly amended SSL soil compared with nominal target concentration; The remaining recovery values show percent change from the initial analytically determined concentration in freshly amended soil during the 8-month study. Measured concentrations are based on USEPA Method 8330A; Values are means (n=3) and Standard Deviations (SD).

4.10. Analytical determinations of HMX in soil treatments used in definitive toxicity tests

Nominal HMX concentrations selected for the composite definitive soil invertebrate or terrestrial plant toxicity tests with TSL soil were 0 (negative control), 0’ (acetone control), 100, 1000, 5000, and 10000 mg/kg. The respective analytically determined HMX concentrations (after the three-month weathering-and-aging in TSL) at the start of the tests were 0, 0’, 72, 913, 4888, and 10208 mg/kg (Table 17). These results indicate that the recovery of HMX was between 98 and 122 percent after the three-month weathering-and-aging in soil.

Table 17. Nominal and analytically determined concentrations of HMX at the beginning and end of the weathering-and-aging process in Teller sandy loam (TSL2002) soil.

Nominal concentration	Analytically determined concentration before weathering-and-aging (mg/kg)	Analytically determined concentration after weathering-and-aging (mg/kg)	Recovery after weathering-and-aging (%)
Negative control	BDL	BDL	NA
Carrier control	BDL	BDL	NA
100	70 ± 15	72 ± 44	103
1000	933 ± 131	913 ± 133	98
5000	4017 ± 35	4888 ± 328	122
10000	8833 ± 522	10208 ± 1143	116

Table notes: BDL= below analytical detection limit (0.34 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

Results of the statistical analyses of the earthworm *Eisenia fetida* reproduction portion of the test were inconclusive. Therefore, an additional definitive test was performed using nominal HMX concentrations 0, 0', 5, 10, 20, 40, 60, 80, 100, 200, and 400 mg/kg. Analytical determinations of HMX in this earthworm definitive test are presented in Table 18. Recovery of HMX was between 88 and 125 percent after the three-month weathering-and-aging in soil.

Table 18. Nominal and analytically determined concentrations of HMX at the beginning and end of the weathering-and-aging process in Teller sandy loam (TSL2002) soil used for the earthworm repeat toxicity test.

Nominal concentration	Analytically determined concentration after weathering-and-aging (mg/kg)	Recovery after weathering-and-aging (%)
Negative control	BDL	NA
Carrier control	BDL	NA
5	5 ± 0.1	94
10	9 ± 0.1	88
20	18 ± 0.3	92
40	39 ± 1.2	97
60	57 ± 0.3	95
80	79 ± 0.1	99
100	98 ± 1	98
200	187 ± 26	93
400	499 ± 15	125

Table notes: BDL= below analytical detection limit (0.34 mg/kg); NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.11. Analytical determinations of NG in soil used in the range-finding toxicity tests

Nominal concentrations selected for range-finding toxicity tests with nitroglycerin (NG) were 0, 0', 1, 10, 100, 1000, and 5000 mg/kg dry SSL2004 soil. The measured concentrations of NG at the beginning of the toxicity tests are presented in Table 19. The measured concentration was usually quite on target (> 84% of the nominal concentration) except for 10 mg/kg, for which the measured concentration was 49% of the nominal concentration. The technical difficulty of weighing small quantity of NG may explain this discrepancy.

Table 19. Nominal and analytically determinations of NG in SSL soil at the beginning of the range-finding toxicity tests.

Nominal concentration (mg/kg)	Measured concentration (mg/kg)			Recovery (%)
Negative control	BDL			NA
Carrier control	BDL			NA
1	0.8	±	0.02	84
10	4.9	±	0.2	49
100	85	±	3	85
1000	898	±	97	90
5000	4558	±	119	91

Table notes: BDL= below analytical detection limit (0.5 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.12. Pilot tests of NG stability in SSL or WCL soil

Two soils (SSL2004 and WCL2001) were individually amended at General Dynamics with nitroglycerin to achieve nominal concentrations of 10, 100, and 5000 mg NG/kg soil. Actual values as measured at the time of soil amendment (To) were 11 ± 0.5, 82 ± 1.3, and 4972 ± 214 mg NG/kg soil for SSL soils, and 11 ± 0.1, 88 ± 1.4, and 5571 ± 279 mg NG/kg soil for WCL soil (Figures 3, 4, and 5). Soils (SSL2004 and WCL2001) individually amended to achieve concentrations of 100 mg/kg had analytically determined concentrations 20% below target. The recovery of NG was confirmed by using an internal standard (HMX), and its recovery ranged from 87-103%.

There was a considerable decrease in NG concentrations in WCL soils. The NG concentrations in the lowest treatment tested (10 mg NG/kg soil) decreased by 50% after 14 d and by 90% after 21 d. After 42 d, the concentrations of NG were below the detection limit (BDL = 0.5 mg/kg). In the next highest concentration tested (100 mg NG/kg soil), the decrease in NG concentrations was 34% after 14 d, 51% after 21 d, and 80% after 42 d. However in the highest concentration tested (5000 mg NG/kg soil), NG remained stable. For the SSL soils, the NG concentrations remained stable until 42 d, when the concentrations decreased considerably, 85% for the lowest concentration (10 mg NG/kg soil) and 59% for the next concentration (100 mg/kg soil). At the highest treatment of 5000 mg NG/kg soil, the concentration remained stable.

The NG concentrations were determined in SSL soil samples that were kept frozen at –20°C for approximately 5 months. Preliminary results showed that the concentrations of NG measured at the beginning of the tests (T0) remained stable in the lowest and highest treatments (10 mg NG/kg soil and 5000 mg NG/kg soil, respectively). The intermediate treatment (100 mg NG/kg) showed a 16% decrease in the NG concentrations (Figures 6, 7, and 8).

These preliminary results showed that NG at 100 mg/kg soil or lower was less stable in WCL soil compared to SSL soil. This could be attributed to biotic or abiotic degradation, formation of NG metabolites, binding to soil constituents, or becoming resistant to extraction due to other fate process in soil. Further studies are needed to better understand the fate of NG in soil.

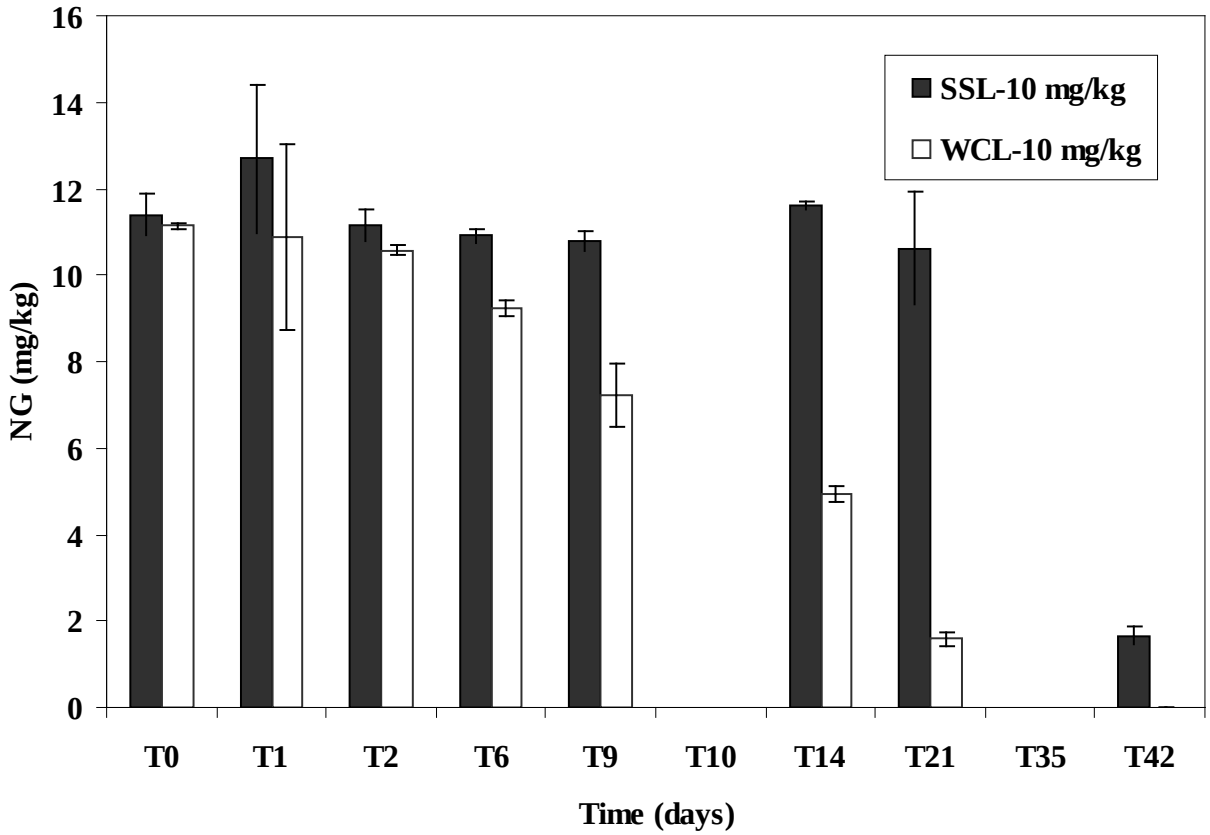


Figure 3. NG stability in Sassafras sandy loam (SSL) and Webster clay loam (WCL) soils individually amended with 10 mg/kg nominal concentration. (BDL: below detectable level ≤ 0.5 mg/kg).

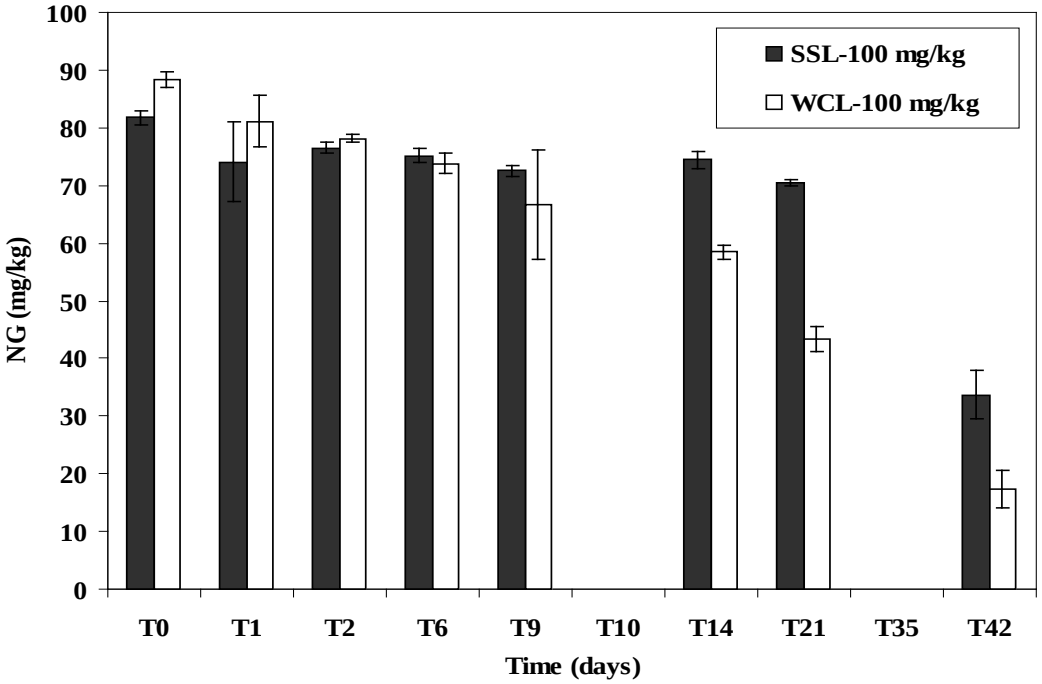


Figure 4. NG stability in Sassafras sandy loam (SSL) and Webster clay loam (WCL) soils individually amended with nominal 100 mg/kg concentration.

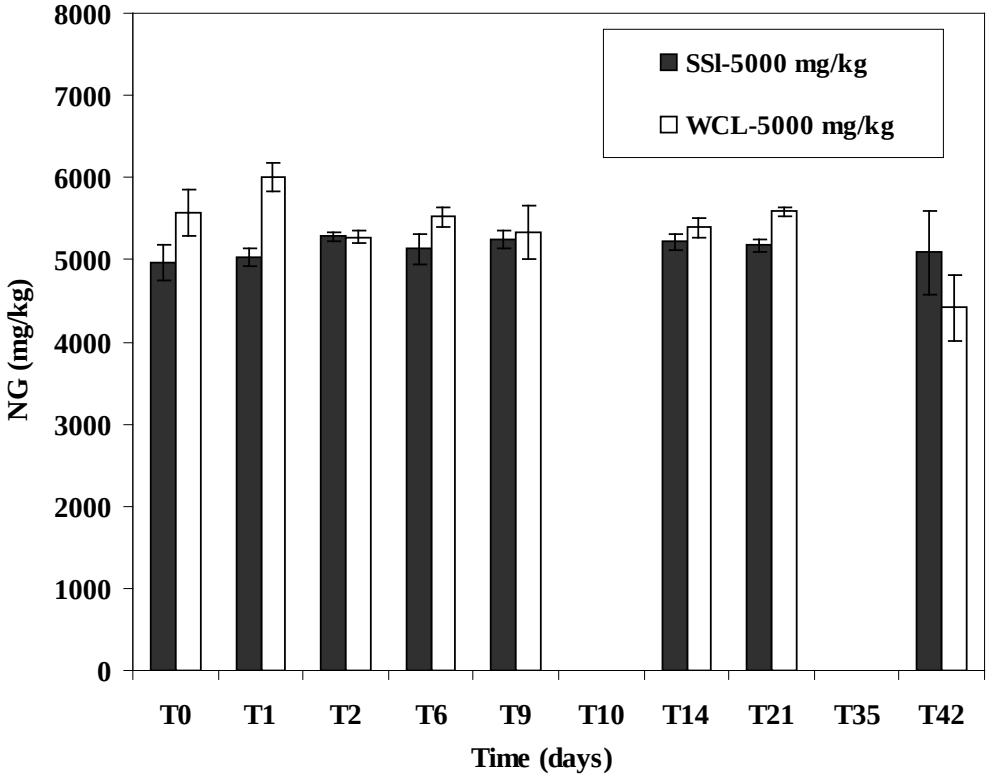


Figure 5. NG stability in Sassafras sandy loam (SSL) and Webster clay loam (WCL) soils individually amended with nominal 5000 mg/kg concentration.

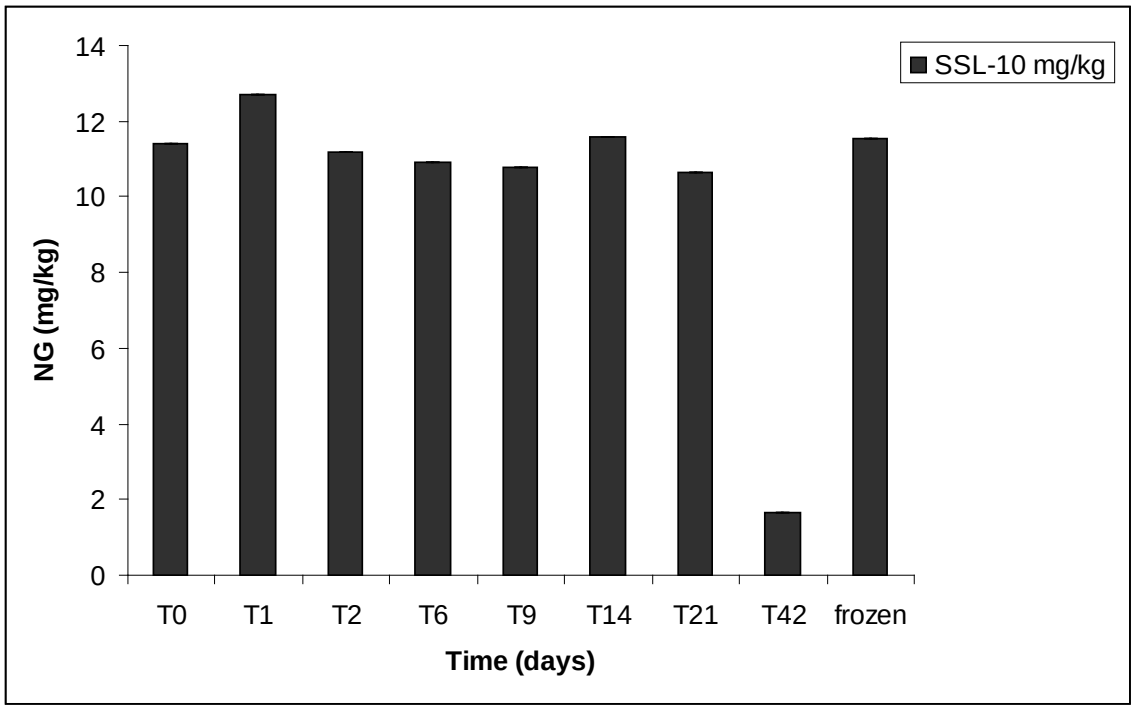


Figure 6. NG stability in Sassafras sandy loam (SSL) soil amended with nominal 10 mg/kg concentration. An aliquot of the To sample was stored at –20°C for 5 months and thawed for analysis.

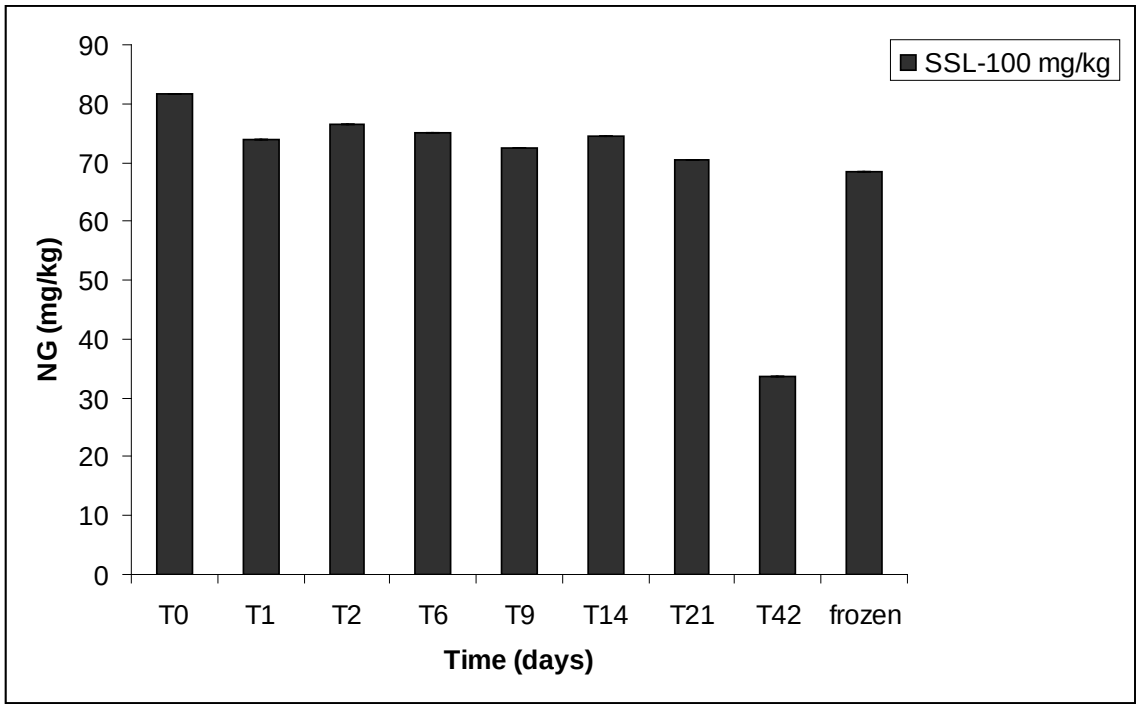


Figure 7. NG stability in Sassafras sandy loam (SSL) soil amended with nominal 100 mg/kg concentration. An aliquot of the To sample was stored at –20°C for 5 months and thawed for analysis.

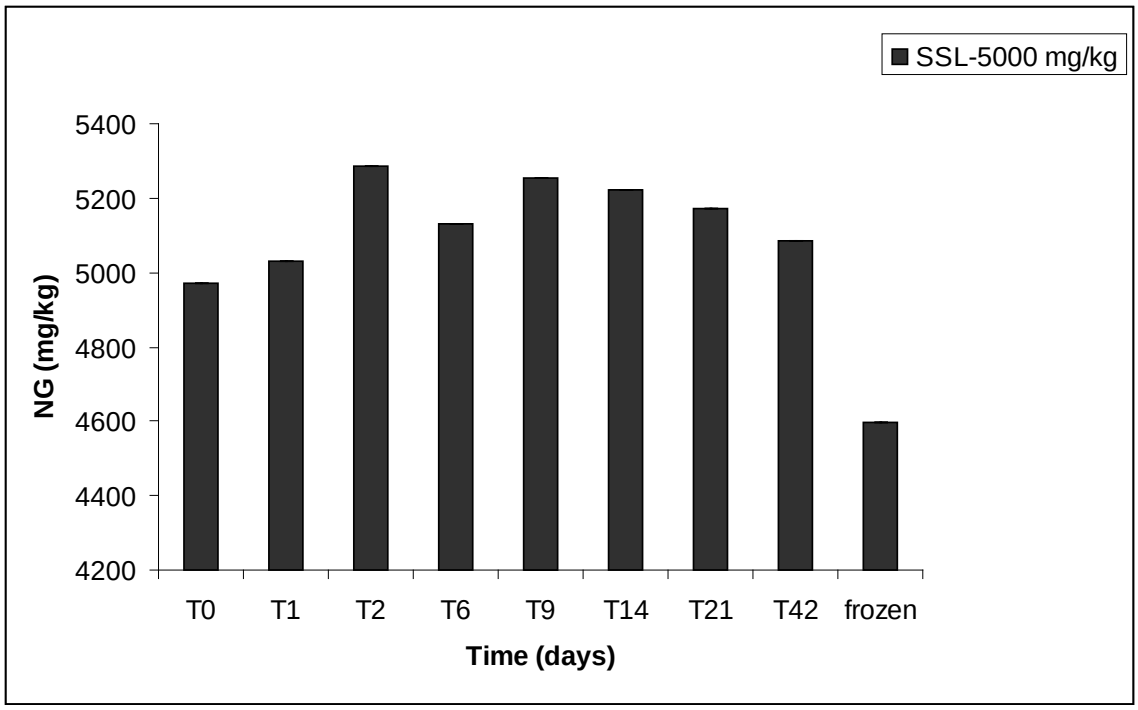


Figure 8. NG stability in Sassafras sandy loam (SSL) amended with nominal 5000 mg/kg concentration. An aliquot of the To sample was stored at –20°C for 5 months and thawed for analysis.

4.13. Pilot weathering-and-aging of NG in SSL2007d soil

A pilot study of weathering-and-aging of NG in the SSL2007d soil was initiated prior to the definitive toxicity tests. Analytical determinations of NG measured at the beginning, after 1 and 2 months of the weathering-and-aging process, are presented in Table 20. Analytically determined NG concentrations at the beginning of the weathering-and-aging process were comparable to nominal concentrations for treatments greater than and equal to 100 mg/kg, with percentages of recovery ranging from 92 to 106. For the nominal concentrations of 5 and 10 mg/kg, these percentages were 184 and 121, respectively. The technical difficulty of weighing small quantities of NG may explain this discrepancy. Due to its rapid degradation, as indicated in Table 20, NG was weathered-and-aged in SSL2007d soil for only 30 days.

Table 20. Nominal and analytically determined concentrations of NG during the pilot weathering-and-aging (W/A) study in Sassafras sandy loam (SSL2007d) soil.

Nominal concentration (mg/kg)	Measured concentration at To (mg/kg)	Recovery Measured/Nominal (%)
0	BDL	NA
5	9.2 ± 0.6	184
10	12 ± 0.8	121
100	92 ± 3	92
200	202 ± 6	101
500	527 ± 34	106
800	797 ± 37	100
Nominal concentration (mg/kg)	**Measured concentration after1 month of W/A (mg/kg)**	**Recovery Measured/To (%)**
0	BDL	NA
5	0.9 ± 0.2	10
10	1 ± 0.3	8
100	15 ± 1	17
200	66 ± 2	33
500	336 ± 8	64
800	629 ± 157	79
Nominal concentration (mg/kg)	**Measured concentration after 2 months of W/A (mg/kg)**	**Recovery Measured/To (%)**
0	BDL	NA
5	BDL	0
10	BDL	0
100	6 ± 0.4	6
200	34 ± 1	17
500	258 ± 6	49
800	446 ± 17	56

Table notes: BDL= below analytical detection limit (0.5 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.14. Analytical determinations of NG weathered-and-aged in SSL soil treatments used in definitive toxicity test with soil invertebrates and terrestrial plants

Analytical determinations of NG in SSL2007d soil treatments are presented in Table 21. Results indicate that the recovery of NG was between 0 and 41 percent after the one-month weathering-and-aging in soil. Traces of a NG metabolite (1,2-DNG) were detected in nominal concentrations at and above 200 mg/kg.

Table 21. Nominal and analytically determined concentrations of NG at the beginning and end of the one-month weathering-and-aging process in Sassafras sandy loam (SSL2007d) soil.

Nominal concentration	Analytically determined concentration before weathering-and-aging (mg/kg)	Analytically determined concentration after weathering-and-aging (mg/kg)	Recovery after weathering-and-aging (%)
Negative control	BDL	BDL	NA
Carrier control	BDL	BDL	NA
2	1.7 ± 0.2	BDL	0
5	3.7 ± 0.4	BDL	0
10	6.9 ± 0.2	BDL	0
20	17 ± 1.5	0.2 ± 0.3	1
30	31 ± 1.6	1.3 ± 0.2	4
50	48 ± 0.4	0.6 ± 0.3	1
75	67 ± 0.2	2.1 ± 0.1	3
100	96 ± 2	1.8 ± 0.1	2
140	124 ± 3	2.4 ± 0.1	2
160	160 ± 2	6.2 ± 0.4	4
200	204 ± 6	21 ± 1	10
250	242 ± 2	22 ± 0	9
300	299 ± 6	36 ± 1	12
400	404 ± 8	122 ± 3	34
650	673 ± 14	268 ± 4	40

Table notes: BDL= below analytical detection limit (0.5 mg/kg). NA=Not Applicable; Values are means (n=3) and Standard Deviations (SD).

4.15. Analytical determinations of NG in SSL soil during the litter decomposition study

Concentrations of NG were analytically determined in each freshly amended treatment of SSL2007e soil to establish a baseline of initial concentrations for litter decomposition study. The initial concentration of NG in the lowest treatment was only 29% of nominal 100 mg/kg treatment. This low recovery rate suggests rapid biotransformation processes in freshly amended soil during the short (2-3 days) period in transit to analytical lab. The remaining initial analytically determined concentrations of NG were generally consistent with the respective target concentrations of 1000, 5000, and 10,000 mg/kg (Table 22). Concentrations of NG in each treatment were analytically determined on each straw harvest date during the eight-month study. Percent recovery declined steadily over time in all nominal treatments, but at a greater rate in the lowest two nominal treatments of 100 and 1000 mg/kg, providing indirect evidence of inhibition of microbial activity at 5000 and 10,000 mg/kg treatments (Table 22).

Table 22. Nominal and analytically determined concentrations of NG in Sassafras sandy loam (SSL2007e) soil during the eight-month litter decomposition study with *Dactylis glomerata*.

Nominal concentration	100		1000		5000		10,000	
	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %
Measured Initial	29 (1)	29[†]	950 (48)	95[†]	5000 (209)	100[†]	10000 (46)	100[†]
1 Month	3.3 (0.1)	11	620 (33)	66	4639 (216)	94	8797 (538)	88
2 Months	1.1 (0.01)	4	481 (12)	51	3879 (84)	78	8203 (388)	82
3 Months	0.6 (0.2)	2	340 (7)	36	3642 (254)	74	7525 (525)	75
4 Months	0	0	350 (2)	37	4169 (81)	84	8038 (352)	80
6 Months	0.7 (0.1)	2	273 (8)	29	3663 (236)	74	7367 (320)	74
8 Months	0	0	219 (13)	23	3697 (56)	75	8005 (791)	80

Table notes: [†]Percent initial recovery of NG from freshly amended SSL soil compared with nominal target concentration; The remaining recovery values show percent change from the initial analytically determined concentration in freshly amended soil during the 8-month study. Measured concentrations are based on USEPA Method 8330A; Values are means (n=3) and Standard Deviations (SD).

4.16. Analytical determinations of selected EMs in SSL at the beginning of the soil enzyme assays

Concentrations of EMs were analytically determined in each freshly amended treatment of SSL2007e soil to establish the initial measured concentrations for the soil enzymes assays. The same batch of soil was used for litter decomposition and soil enzymes assays. Therefore initial concentrations stated in Tables 9, 13, and 16 apply for 2-ADNT, 4-ADNT, and 2,4-DNT. For nitroglycerine (NG), there was a delay (more than 1 week) between litter decomposition and soil enzymes testing, and potential nitrification (PN) assays were repeated to meet quality control criteria. The NG concentrations used for soil enzyme assays are presented in Table 23. The initial concentration of NG in the lowest treatment was 27% of nominal 10 mg/kg treatment, and 61-64% of nominal 100 mg/kg treatment. These low recovery rate suggests rapid biotransformation processes in freshly amended soil during the short (1-2 days) period in transit to analytical lab. The remaining initial analytically determined concentrations of NG were consistent with the respective target concentrations of 1000, 5000, and 10000 mg/kg, and showed 89 % recovery and higher (Table 23).

Table 23. Nominal and analytically determined concentrations of nitroglycerin (NG) in Sassafras sandy loam (SSL2007e) soil at the beginning of the soil enzymes assays.

Nominal concentration	10		100		1000		5000		10000	
	Mean (SD) mg/kg	Recovery† %	Mean (SD) mg/kg	Recovery† %	Mean (SD) mg/kg	Recovery† %	Mean (SD) mg/kg	Recovery† %	Mean (SD) mg/kg	Recovery† %
1st set of measured NG	nd	nd	64 (0.7)	63	974 (39)	97	5775 (339)	116	9785 (869)	98
2nd set of measured NG	2.7 (0.1)	27	61 (1.4)	61	990 (63)	99	4639 (226)	93	8929 (327)	89

Table notes: †Percent recovery of NG from freshly amended SSL soil compared with nominal target concentration. nd: Not determined. Measured concentrations are based on USEPA Method 8330A; Values are means (n=3) and Standard Deviations (SD). The 1st set of measured NG was used for APA, DH, and NAG assays, while the 2nd set of measurements was used for PN assay.

5. Effects of energetic material on terrestrial plants

5.1. Phytotoxicity of the positive control (boric acid)

A positive chemical control is required in toxicity testing with terrestrial plants to validate the condition of the test species, and reliability and precision of results. Toxicity tests with a reference toxicant, boric acid (H_3BO_3), were conducted with alfalfa (*Medicago sativa* L.), barnyard grass (also referred to as Japanese millet in some publications) (*Echinochloa crusgalli* (L). Beauv.), and perennial ryegrass (*Lolium perenne* L.) using the seed stocks of 2005.

In 2005 and 2006, the toxicity of boric acid was assessed in the SSL2004 soil, while the SSL2007d soil was used in 2007. Some indigenous seeds present in the SSL2007d soil germinated in the test pots and their seedlings were very similar to the ryegrass Express seedlings. The physical differences between the species are presented in Figure 9. The stem of ryegrass Express seedlings has two leaves of unequal length in contrast to the indigenous species, which have either a stem with three leaves or a stem with two leaves of similar length.

Experimentally determined boric acid EC_{50} values for seedling emergence and shoot growth are presented in Table 24. Seedling emergence was greater than 80% for the three plant species, and toxicity endpoint values complied with quality control criteria over the three-year project period.

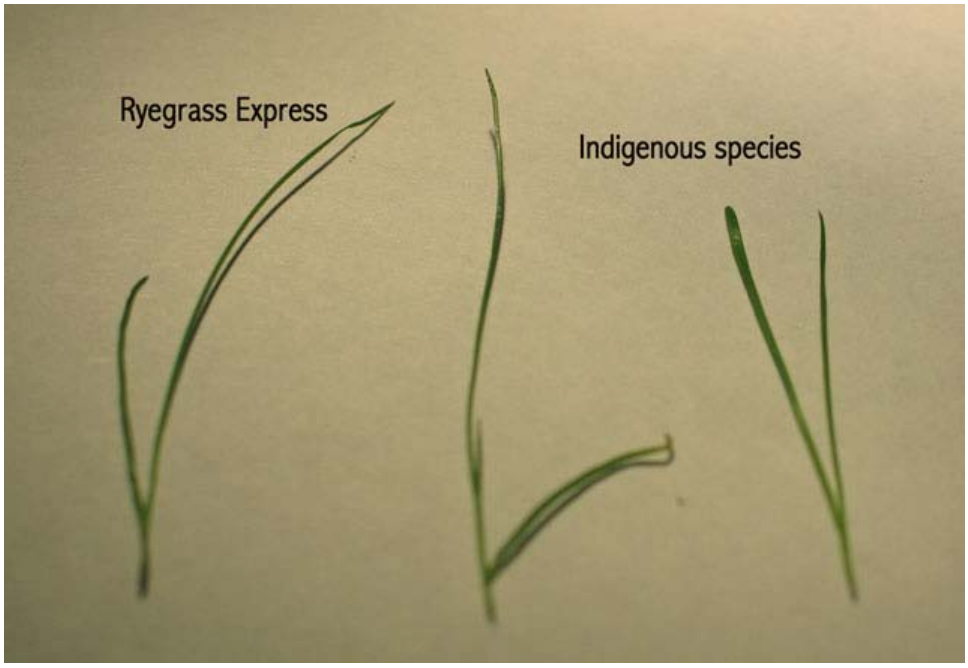


Figure 9. Pictures of ryegrass Express and two indigenous species after 19 days of growth.

Table 24. Boric acid test parameters for alfalfa, barnyard grass, and perennial ryegrass established in Sassafras sandy loam soil during the 2005-2007 testing period.

Ecotoxicological parameters	Alfalfa *	Barnyard grass	Ryegrass
2007 test			
Seedling emergence EC_{50} (mg/kg)	188	382	162
Confidence intervals (95%)	154-239	348-481	125-178
Shoot growth dry mass EC_{50} (mg/kg)	120	75	76
Confidence intervals (95%)	99-156	30-113	63-91
Seedling emergence in negative control (%)	82	87	92
Mean shoot mass in negative control (g)	0.028	0.048	0.053
Minimum and maximum values	0.021-0.035	0.033-0.056	0.046-0.061
2006 test			
Seedling emergence EC_{50} (mg/kg)	>290	>450	118
Confidence intervals (95%)	---	---	96-160
Shoot growth dry mass EC_{50} (mg/kg)	199	79	77
Confidence intervals (95%)		38-114	55-94
Seedling emergence in negative control (%)	82	83	95
Mean shoot mass in negative control (g)	0.030	0.065	0.060
Minimum and maximum values	0.022-0.036	0.037-0.081	0.054-0.066
2005 test			
Seedling emergence EC_{50} (mg/kg)	224	305	148
Confidence intervals (95%)	200-304	192-422	126-179
Shoot growth dry mass EC_{50} (mg/kg)	135	70	74
Confidence intervals (95%)	100-173	51-88	64-83
Seedling emergence in negative control (%)	85	93	87
Mean shoot mass in negative control (g)	0.038	0.074	0.056
Minimum and maximum values	0.0.36-0.040	0.067-0.081	0.054-0.058

Table note: * Alfalfa seeds were inoculated with nitrogen-fixing bacteria prior to sowing.

5.2. Phytotoxicity of 2,4-DNT

Definitive plant toxicity tests of 2,4-DNT weathered-and-aged in three natural soils, Teller sandy loam (TSL), Kirkland clay loam (KCL) or Kirkland loam (KL) (See Section 4.2.2.2), and Webster clay loam (WCL) were conducted using alfalfa, barnyard grass, and ryegrass. Treatment concentrations of 2,4-DNT in each soil were prepared as single batches for toxicity studies. Tests were performed using four replicates of each soil treatment and the three test species. The 2,4-DNT was weathered-and-aged in soil for a period of 3 months.

5.2.1. Effects of 2,4-DNT weathered-and-aged in TSL soil on terrestrial plants

Nominal concentrations of 2,4-DNT in TSL2002 soil were 2, 5, 10, 20, 40, 80, 160, and 300 mg/kg for alfalfa; 2, 5, 10, 20, 40, 60, 80, and 160 mg/kg for barnyard grass; and 0, 0', 2, 5, 10, 15, 20, 40, and 60 mg/kg for ryegrass. Water and carrier (acetone) controls were included in these phytotoxicity tests. Analytically determined concentrations of 2,4-DNT at the beginning of the phytotoxicity tests are presented in Table 4.

Seedling emergence of alfalfa, barnyard grass, and ryegrass in the carrier control was 80, 86, and 96%, respectively, which complies with the quality control criterion for this endpoint.

Toxicological parameters for 2,4-DNT weathered-and-aged in TSL2002 determined in the definitive toxicity tests with alfalfa, barnyard grass, and ryegrass are summarized in Table 25. The logistic (Gompertz) model had the best fit for seedling emergence and shoot growth (fresh and dry mass; Figure 10). Values for regression coefficients determined for all ECp endpoints were greater than 0.95, indicating a good fit of the model used for toxicity data. EC_{20} values (mg/kg) for shoot growth (dry mass) were 7 (0-15, 95% CI), 9 (2-15, 95% CI), and 8 (6-11, 95% CI) for alfalfa, barnyard grass, and ryegrass, respectively (Table 25). These results were significantly different from the earlier results obtained in 2006 for alfalfa (65 mg/kg; 14-116, 95% CI) but not significantly different for barnyard grass (13 mg/kg; 3-23, 95% CI) and ryegrass (8 mg/kg; 6-9, 95% CI).

Table 25. Summary of toxicological parameters for 2,4-DNT (mg/kg) weathered-and-aged in Teller sandy loam determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	26[†]	43	10
p	0.292	0.568	1.000
LOEC	62[‡]	62	15
p	<0.001	0.018	0.004
EC_{20}	30	112	15
Confidence intervals (95%)	4-55	59-166	14-17
EC_{50}	94	>118	19
Confidence intervals (95%)	57-130	ND	18-20
Model used	Gompertz	Gompertz	Gompertz
R^2	0.959	0.987	0.994
Growth - Fresh mass			
NOEC	3	6	6
p	0.092	0.619	0.127
LOEC	6	15	10
p	0.024	<0.001	<0.001
EC_{20}	5	9	6
Confidence intervals (95%)	1-8	5-13	5-8
EC_{50}	26	15	11
Confidence intervals (95%)	16-37	12-19	10-12
Model used	Gompertz	Gompertz	Gompertz
R^2	0.973	0.961	0.984
Growth - Dry mass			
NOEC	6	15	6
p	0.087	0.170	0.675
LOEC	15	26	10
p	<0.001	<0.001	<0.001
EC_{20}	7	9	8
Confidence intervals (95%)	0-15	2-15	6-11
EC_{50}	44	26	12
Confidence intervals (95%)	22-66	17-35	10-14
Model used	Gompertz	Gompertz	Gompertz
R^2	0.964	0.953	0.964

Table notes: Concentrations are based on USEPA Method 8330A; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.
[†] NOAEC: No observable adverse effect concentration
[‡] LOAEC: Lowest observable adverse effect concentration

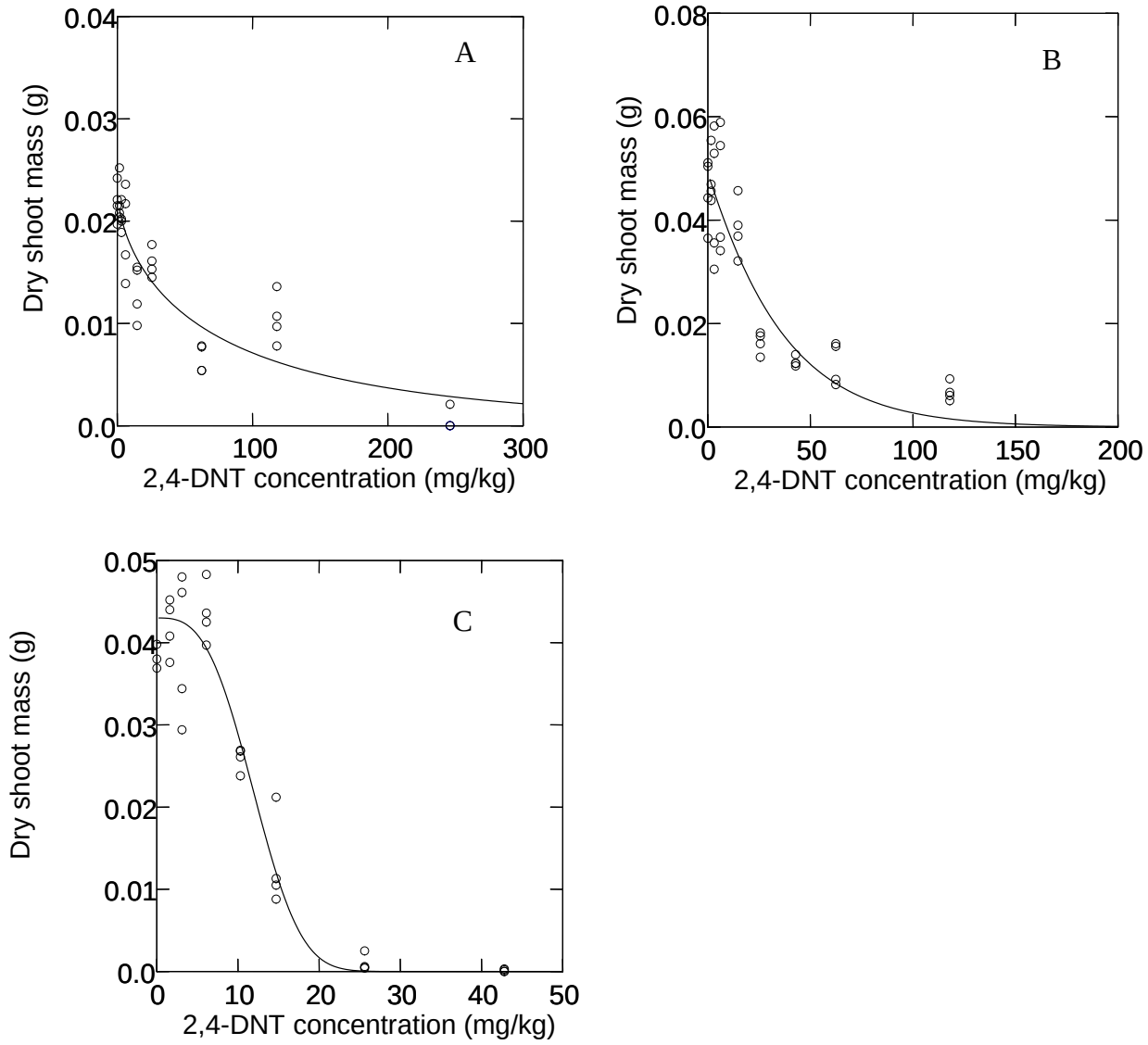


Figure 10. Effects of 2,4-DNT weathered-and-aged in Teller sandy loam (TSL2002) soil on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth.

5.2.2. Effects of 2,4-DNT weathered-and-aged in Kirkland soil on terrestrial plants

Nominal concentrations of 2,4-DNT in Kirkland soil were 2, 5, 10, 20, 40, 80, 160, 300, and 600 mg/kg for alfalfa and barnyard grass; and 2, 5, 10, 15, 20, 40, 60, and 160 mg/kg for ryegrass. Water and carrier (acetone) controls were included in these phytotoxicity tests. Indigenous alfalfa seeds were present in the KCL2002 soil, which interfered with the growth of our alfalfa seed stock. Therefore, the alfalfa definitive toxicity test was repeated using the KL2006 soil. Analytically determined concentrations of 2,4-DNT in KCL2002 are presented in Table 6, and concentrations of 2,4-DNT in KL2006 soil are presented in Table 7. Results showed that after

the three-month weathering-and-aging of 2,4-DNT in soil, 2,4-DNT concentrations decreased by 41 to 65% in the KCL2002 soil, and by 64 to 81% in the KL2006 soil compared to the initial concentrations in freshly amended soil.

Seedling emergence of alfalfa, barnyard grass, and ryegrass in the carrier control was 85, 79, and 83%, respectively, which complies with the quality control criterion for this endpoint.

Toxicological parameters for 2,4-DNT weathered-and-aged in KCL2002 or KL2006 are summarized in Table 26. The EC_{20} or EC_{50} values for barnyard grass seedling emergence could not be determined within the 2,4-DNT concentration range tested in KCL2002. The EC_{20} values for seedling emergence of alfalfa and ryegrass were 71 mg 2,4-DNT/mg dry KL soil and 14 mg 2,4-DNT/mg dry KCL soil, respectively. The range of 2,4-DNT concentrations selected was sufficient to establish the concentration-response relationships for growth measurement endpoint (dry shoot mass) for each species tested (Figure 11). The EC_{20} values for shoot growth (dry mass) were 40 mg 2,4-DNT/mg dry KL soil, 5 mg 2,4-DNT/mg dry KCL soil and 10 mg 2,4-DNT/mg dry KCL soil, for alfalfa, barnyard grass and ryegrass, respectively. Results showed that barnyard grass was the most sensitive species to 2,4-DNT compared to alfalfa or ryegrass, based on the shoot growth (dry mass) endpoint and respective EC_{20} values.

Table 26. Summary of toxicological parameters for 2,4-DNT (mg/kg) weathered-and-aged in Kirkland soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Soil	KL2006	KCL2002	KCL2002
Seedling emergence			
NOEC	56	87	9
p	0.079	0.850	0.751
LOEC	89	175	18
p	0.001	<0.001	<0.001
EC_{20}	71	>175	14
Confidence intervals (95%)	51-91	ND	8-19
EC_{50}	141	>175	16
Confidence intervals (95%)	117-164	ND	14-19
Model used	Gompertz	ND	Gompertz
R^2	0.987	ND	0.995
Growth - Fresh mass			
NOEC	7 †	<1	7 †
p	0.429	ND	0.766
LOEC	14 ‡	1	9 ‡
p or P(T<=t) two-tail	<0.001	0.015	0.001
EC_{20}	21	3	7
Confidence intervals (95%)	7-35	2-4	6.5-8
EC_{50}	46	11	9
Confidence intervals (95%)	31-60	9-12	8-10
Model used	Gompertz	Exponential	Hormetic
R^2	0.942	0.962	0.981
Growth - Dry mass			
NOEC	7 †	9	9
p	0.488	0.539	0.588
LOEC	14 ‡	18	18
p or P(T<=t) two-tail	0.005	<0.001	<0.001
EC_{20}	40	5	9.6
Confidence intervals (95%)	25-55	4-7	9-11
EC_{50}	65	16	11
Confidence intervals (95%)	54-77	11-20	10-13
Model used	Gompertz	Exponential	Hormetic
R^2	0.962	0.971	0.989

Table notes: Concentrations are based on USEPA Method 8330A; ND=Not Determined: could not be determined within the concentration range tested; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

† NOAEC: No observable adverse effect concentration

‡ LOAEC: Lowest observable adverse effect concentration

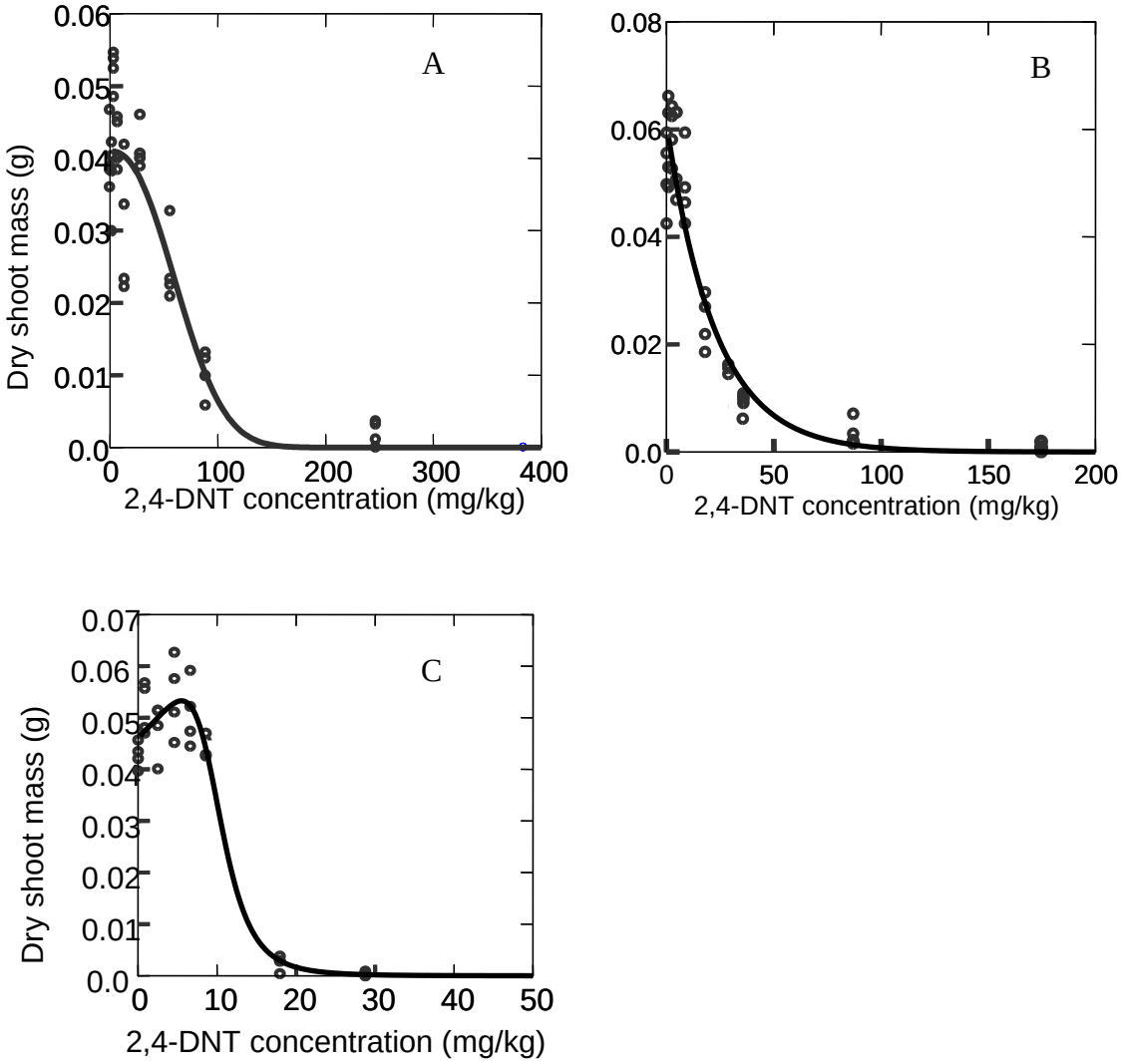


Figure 11. Effects 2,4-DNT weathered-and-aged in Kirkland soil on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth.

5.2.3. Effects of 2,4-DNT weathered-and-aged in WCL soil on terrestrial plants

Nominal concentrations of 2,4-DNT in WCL2001 soil were 2, 5, 10, 20, 40, 80, 160, 300, and 600 mg/kg for alfalfa; 2, 5, 10, 20, 40, 60, 80, 160, and 300 mg/kg for barnyard grass; and 2, 5, 10, 15, 20, 40, 60, and 160 mg/kg for ryegrass. Water and carrier (acetone) controls were included in these phytotoxicity tests. Analytically determined concentrations of 2,4-DNT in WCL2001 are presented in Table 8. Results showed that 2,4-DNT concentrations decreased by 59 to 72% after the three-month weathering-and-aging of 2,4-DNT in WCL2001 soil, compared to the initial concentrations in freshly amended soil . Seedling emergence of alfalfa, barnyard

grass, and ryegrass in the carrier control was 92, 89, and 95%, respectively, which complies with the quality control criterion for this endpoint.

Results presented in Table 27 and Figure 12 show that for alfalfa, barnyard grass and ryegrass, respectively the EC_{20} values for seedling emergence were 258, 197 and 60 mg 2,4-DNT/mg dry WCL soil and the EC_{20} values for shoot growth (dry mass) were 120, 57 and 34 mg 2,4-DNT/mg dry WCL soil. Ryegrass was the most sensitive species for seedling emergence and shoot growth endpoints.

Table 27. Summary of toxicological parameters for 2,4-DNT (mg/kg) weathered-and-aged in Webster clay loam soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	189	<189	39
p	0.110	0.062	0.719
LOEC	447	189	97
p	<0.001	0.783	<0.001
EC_{20}	258	197	60
Confidence intervals (95%)	154-362	ND	41-79
EC_{50}	541	>189	72
Confidence intervals (95%)	378-703	ND	57-88
Model used	Gompertz	Gompertz	Gompertz
R^2	0.987	0.983	0.997
Shoot growth - Fresh mass			
NOEC	28	14	14
p	0.731	0.085	0.083
LOEC	54	28	28
p	0.009	0.008	<0.001
EC_{20}	64	32	22
Confidence intervals (95%)	36-91	26-38	18-25
EC_{50}	157	56	33
Confidence intervals (95%)	122-192	51-62	31-35
Model used	Gompertz	Gompertz	Gompertz
R^2	0.980	0.988	0.994
Shoot growth - Dry mass			
NOEC	97	39	28
p	0.214	0.684	0.310
LOEC	189	54	39
p	<0.001	0.003	<0.001
EC_{20}	120	57	34
Confidence intervals (95%)	74-166	44-69	30-37
EC_{50}	229	84	40
Confidence intervals (95%)	177-280	75-93	39-42
Model used	Gompertz	Gompertz	Gompertz
R^2	0.975	0.984	0.993

Table notes: Concentrations are based on USEPA Method 8330A; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

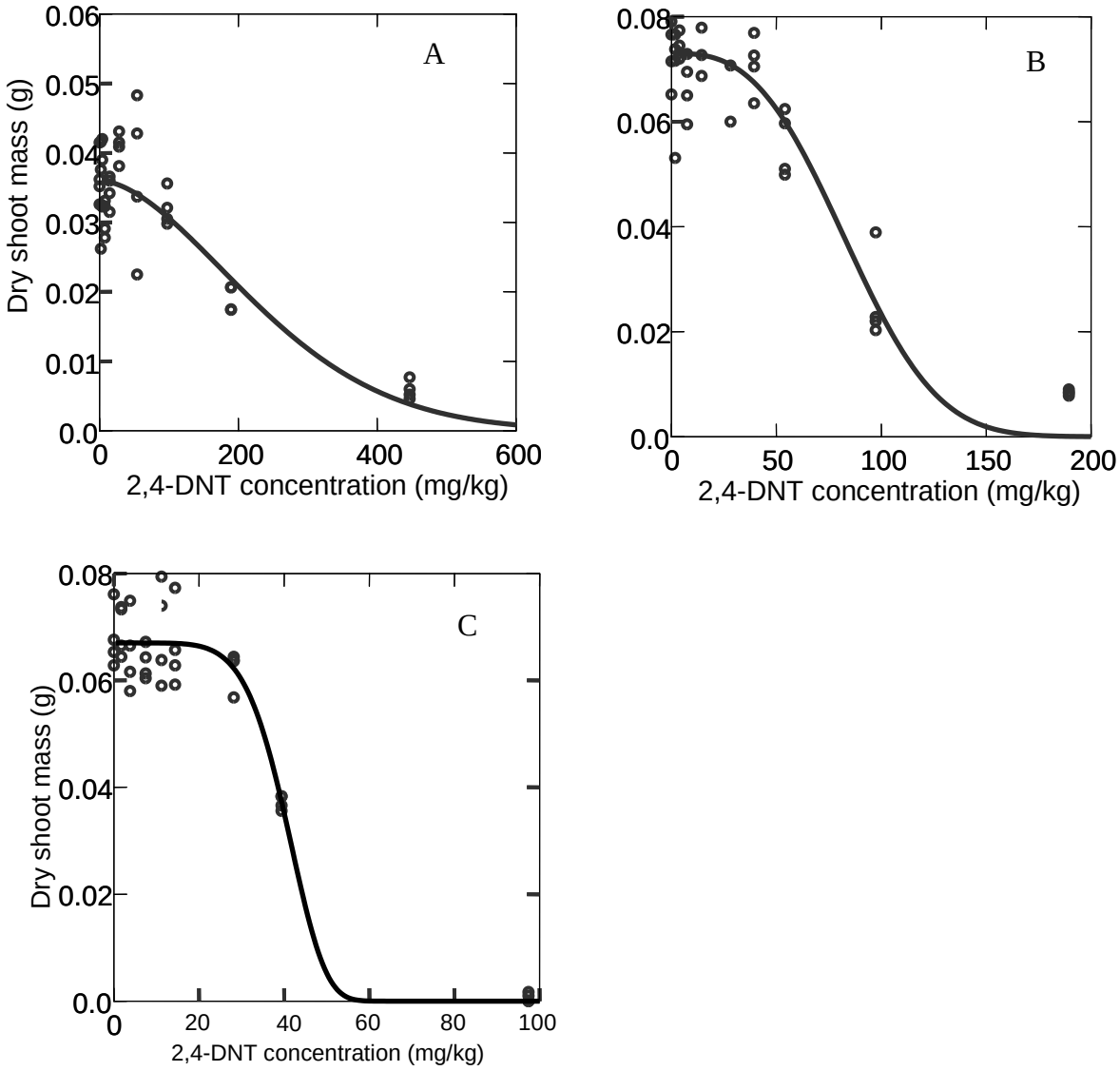


Figure 12. Effects of 2,4-DNT weathered-and-aged in Webster clay loam (WCL2001) soil on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth.

5.2.4. Effects of 2,4-DNT weathered-and-aged in SSL soil on terrestrial plants

Toxicity data for 2,4-DNT established in our studies with SSL soil (corresponding soil batch designations SSL2000) were reported previously in Kuperman *et al.* (2006b) and are included in Table 28 of this report.

Table 28. Summary of toxicological parameters for 2,4-DNT (mg/kg) weathered-and-aged in Sassafras sandy loam soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC or NOAEC[§]	89 [§]	32 [§]	4
p	0.802	0.584	0.586
LOEC or LOAEC [§§]	121 [§§]	91 [§§]	8
p	0.0006	<0.0001	0.014
EC_{20}	104	86	>8
Confidence intervals (95%)	91-117	---	---
EC_{50}	115	>32	>8
Confidence intervals (95%)	109-121	---	---
Model used	Hormetic	Hormetic	---
R^2	0.989	0.994	---
Growth - Fresh mass			
NOEC or NOAEC	6	1	4
p	0.188	0.977	0.498
LOEC or LOAEC	10	4	8
p	<0.0001	0.015	<0.0001
EC_{20}	7	4	5
Confidence intervals (95%)	2-11	2-5	4-7
EC_{50}	30	7	7
Confidence intervals (95%)	20-40	5-8	6-8
Model used	Gompertz	Gompertz	Gompertz
R^2	0.976	0.982	0.992
Growth - Dry mass			
NOEC	6	4	4
p	0.153	0.802	0.421
LOEC	10	8	8
p	0.011	<0.0001	<0.0001
EC_{20}	15	6	2
Confidence intervals (95%)	8.8-21.4	4.9-7.7	0-4.0
EC_{50}	42	10	8
Confidence intervals (95%)	28-56	9-12	---
Model used	Hormetic	Gompertz	Hormetic
R^2	0.979	0.989	0.990

Table notes: Data from Rocheleau *et al.* (2006); Soil concentration of 2,4-DNT (mg/kg) were determined by USEPA Method 8330A; EC=effect concentration; NOEC=no-observed-effect concentration; [§] = NOAEC=no-observed-adverse-effect-concentration; LOEC=lowest-observed-effect concentration; [§§] = LOAEC=lowest-observed-adverse-effect concentration.

5.2.5. Phytotoxicity of 2,4-DNT weathered-and-aged in the different soil types

In all three tested soils, ryegrass (dicot) was generally the most sensitive species, followed by barnyard grass (dicot), and then alfalfa (monocot) grown in symbiosis with nitrogen-fixing Rhizobium bacteria.

Based on the shoot growth endpoint, 2,4-DNT was more toxic in TSL soils followed by KCL soil and WCL soil. Results of correlation analyses for selected soil properties and the EC_{50} toxicity benchmarks for shoot growth (dry mass) determined in the definitive tests with the three plant species are presented in Table 29. Organic matter content of the soil was strongly ($r \geq 0.954$) and significantly ($p < 0.05$) correlated with alfalfa or ryegrass shoot growth toxicity benchmarks for 2,4-DNT weathered-and-aged in soil. Organic matter content of the soil was also strongly ($r = 0.926$) correlated with barnyard grass shoot growth toxicity benchmarks for 2,4-DNT weathered-and-aged in soil but at a greater p-value of 0.074. Soil clay content explained between 45 and 61 percent in variance of shoot growth toxicity benchmarks for 2,4-DNT, however, none of the correlations were statistically significant at p=0.05. No significant ($p \geq 0.420$) correlations were found among the EC_{50} phytotoxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter as the dominant property mitigating 2,4-DNT toxicity for the three plant species tested in the present studies.

Table 29. Correlation coefficients for key soil properties and EC_{50} benchmarks for shoot growth (dry mass) determined in the definitive tests with Alfalfa, Barnyard grass, and Ryegrass exposed to 2,4-DNT weathered-and-aged in Teller sandy loam, Sassafras sandy loam, Kirkland clay loam, and Webster clay loam soils.

Soil property	Alfalfa shoot growth (EC_{50})	*p-value*	Barnyard grass shoot growth (EC_{50})	*p-value*	Ryegrass shoot growth (EC_{50})	*p-value*
Organic matter	0.976	0.024*	0.926	0.074	0.954	0.046*
Clay	0.613	0.387	0.450	0.550	0.523	0.477
pH	0.420	0.580	0.223	0.777	0.310	0.690

Table note: * Statistically significant correlation ($p \leq 0.05$).

5.3. Effects of 2-ADNT on terrestrial plants

5.3.1. Effects of 2-ADNT on terrestrial plants in freshly amended SSL soil

Range-finding plant toxicity tests were conducted in triplicate with 2-ADNT freshly amended into SSL2007d soil. Nominal concentrations selected for these tests were 0 (negative control), 0' (carrier control), 100, 300, 1000, 3000, and 5000 mg/kg. Corresponding analytically determined concentrations of 2-ADNT in SSL2007d soil are presented in Table 12. Analytically determined 2-ADNT concentrations were comparable to nominal concentrations, with recovery ranging from 91 to 101%.

Toxicological parameters for 2-ADNT freshly amended into SSL2007d soil determined in the range-finding toxicity tests with alfalfa, barnyard grass, and ryegrass are summarized in Table 30. The range of 2-ADNT concentrations selected for these tests was sufficient to establish the concentration-response relationships for growth measurement endpoint (dry shoot mass) for each species tested (Figure 13). The EC_{20} values (and corresponding 95 percent CI), mg/kg, for shoot growth dry mass were 16 (0-36), 34 (14-55), and 19 (15-22) for alfalfa, barnyard grass and ryegrass, respectively (Table 30 and Figure 13). These results indicated that alfalfa and ryegrass were more sensitive to 2-ADNT freshly amended into SSL2007d soil compared with barnyard grass.

Table 30. Summary of toxicological parameters for 2-ADNT (mg/kg) freshly amended into Sassafras sandy loam (SSL2007d) soil determined in the range-finding toxicity test with alfalfa, barnyard grass and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	≥4929	≥4929	<91
p	0.295	0.574	<0.001
LOEC	>4929	>4929	91 [1]
p	0.295	0.574	<0.001
EC_{20}	49	183	32
Confidence interval (95%)	0-185	0-756	23-41
EC_{50}	153	569	100
Confidence interval (95%)	0-576	0-2348	71-129
Model used	Exponential	Exponential	Exponential
R^2	0.984	0.992	0.975
Growth - Fresh mass			
NOEC	<91	<91	<91
p	<0.001	0.001	<0.001
LOEC	91 [1]	91 [1]	91 [1]
p or P(T<=t) two-tail	<0.001	0.001	<0.001
EC_{20}	17	25	18
Confidence interval (95%)	10-24	13-37	15-20
EC_{50}	54	77	54
Confidence interval (95%)	32-75	39-114	47-62
Model used	Exponential	Exponential	Exponential
R^2	0.986	0.937	0.994
Growth - Dry mass			
NOEC	<91	91	<91
p	0.027	0.055	<0.001
LOEC	91	286	91 [1]
p or P(T<=t) two-tail	0.027	<0.001	<0.001
EC_{20}	16	34	19
Confidence interval (95%)	0-36	14-55	15-22
EC_{50}	51	107	58
Confidence interval (95%)	0-113	42-171	48-69
Model used	Exponential	Exponential	Exponential
R^2	0.957	0.936	0.989

Table notes: Concentrations are based on USEPA Method 8330A; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

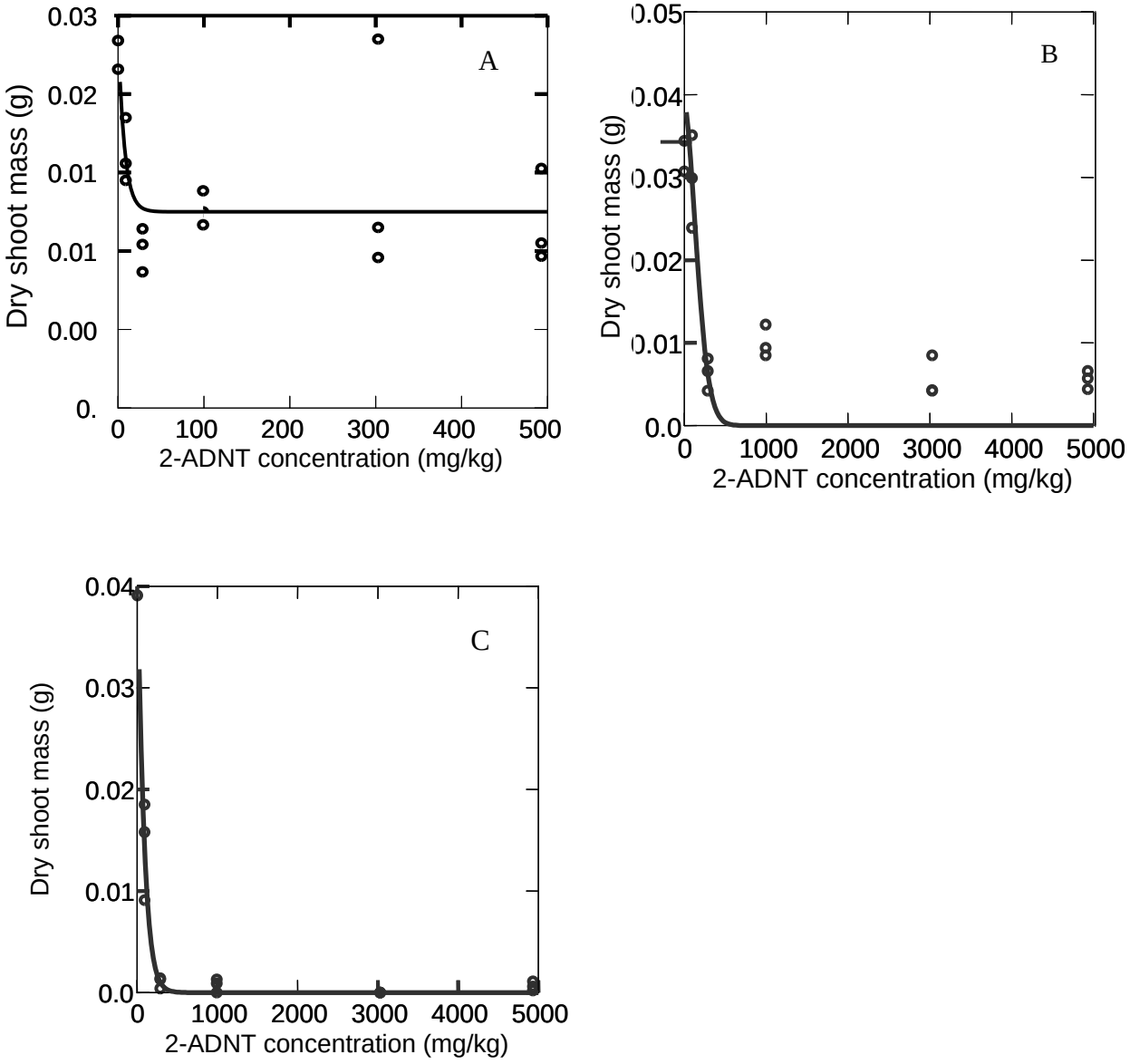


Figure 13. Effects of 2-ADNT on growth (shoot dry mass) of alfalfa (A), barnyard grass (B), and ryegrass (C) in freshly amended Sassafras sandy loam (SSL2007d) soil.

5.3.2. Effects of 2-ADNT weathered-and-aged in soil on terrestrial plants – definitive tests

The effects of weathering-and-aging of 2-ADNT in soil on phytotoxicity were investigated by comparing test results for 2-ADNT weathered-and-aged in SSL with results obtained in freshly amended SSL soil.

Nominal positive exposure concentrations of 2-ADNT in soil for the terrestrial definitive toxicity test were 40, 80, 100, 140, 160, 180, 200, 300, 400, 2000, 5000, and 10000 mg/kg for alfalfa and barnyard grass; and 40, 80, 100, 140, 160, 180, 200, 300, 400, 1000, and 2000 mg/kg for perennial ryegrass. Water and carrier (acetone) controls were included in these phytotoxicity tests. Corresponding analytically determined concentrations of 2-ADNT weathered-and-aged in SSL2007d soil are presented in Table 11. Tests were performed using four replicates of each soil treatment and three test species. 2-ADNT was weathered-and-aged in SSL2007d soil for 3 months.

Seedling emergence of alfalfa, barnyard grass, and ryegrass in the carrier control was 89, 79, and 88 percent, respectively, which complies with the quality control criterion for this endpoint. The logistic (Gompertz) model had the best fit for alfalfa seedling emergence or shoot growth, while the exponential model had the best fit for barnyard grass and ryegrass seedling emergence or shoot growth (fresh or dry mass) (Table 31). Values for regression coefficients determined for all EC_p endpoints were greater than 0.92, indicating a good fit of the model used for toxicity data. The EC_{20} values (and corresponding 95 percent CI), mg/kg, for shoot growth dry mass were 36 (0-134), 9 (5-14), and 22 (17-27) for alfalfa, barnyard grass and ryegrass, respectively (Table 31 and Figure 14).

In order to test the hypothesis that weathering-and-aging 2-ADNT in SSL soil can affect its toxicity to terrestrial plants, the phytotoxicity of 2-ADNT freshly amended into soil was measured, and results were presented in the previous section. The EC_{20} values (and corresponding 95 percent CI), mg/kg, for shoot growth dry mass were 16 (0-36), 34 (14-55), and 19 (15-22) for alfalfa, barnyard grass and ryegrass, respectively (Table 31). Based on these results, the toxicity of freshly amended 2-ADNT was not significantly different from the toxicity of 2-ADNT weathered-and-aged in SSL soil.

Table 31. Summary of toxicological parameters for 2-ADNT (mg/kg) weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	1597	8287	99
p	0.514	0.408	0.479
LOEC	4027	>8287	120
p	0.009	ND	0.004
EC_{20}	4021	>8287	60
Confidence interval	0-12057	ND	38-82
R^2	0.978	ND	0.979
EC_{50}	1203	>8287	186
Confidence interval	0-3887	ND	117-254
Model used	Exponential	ND	Exponential
R^2	0.978	ND	0.979
Growth - Fresh mass			
NOEC	<21	<21	21
p	ND	ND	0.228
LOEC	21 [1]	21 [1]	45
p or P(T<=t) two-tail	0.002	0.002	<0.001
EC_{20}	4	8	27
Confidence interval	0-15	6-11	21-34
R^2	0.948	0.919	0.982
EC_{50}	53	25	51
Confidence interval	35-72	18-33	44-58
Model used	Exponential	Exponential	Exponential
R^2	0.972	0.919	0.980
Growth - Dry mass			
NOEC	<21	<21	58 [2]
p	ND	ND	0.498
LOEC	21 [1]	21 [1]	90 [3]
p or P(T<=t) two-tail	0.012	0.019	0.029
EC_{20}	36	9	22
Confidence interval	0-134	5-14	17-27
EC_{50}	52	29	69
Confidence interval	23-81	17-42	53-85
Model used	Gompertz	Exponential	Exponential
R^2	0.970	0.932	0.955

Table notes: Concentrations are based on USEPA Method 8330A; ND=Not Determined: could not be determined within the concentration range tested; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

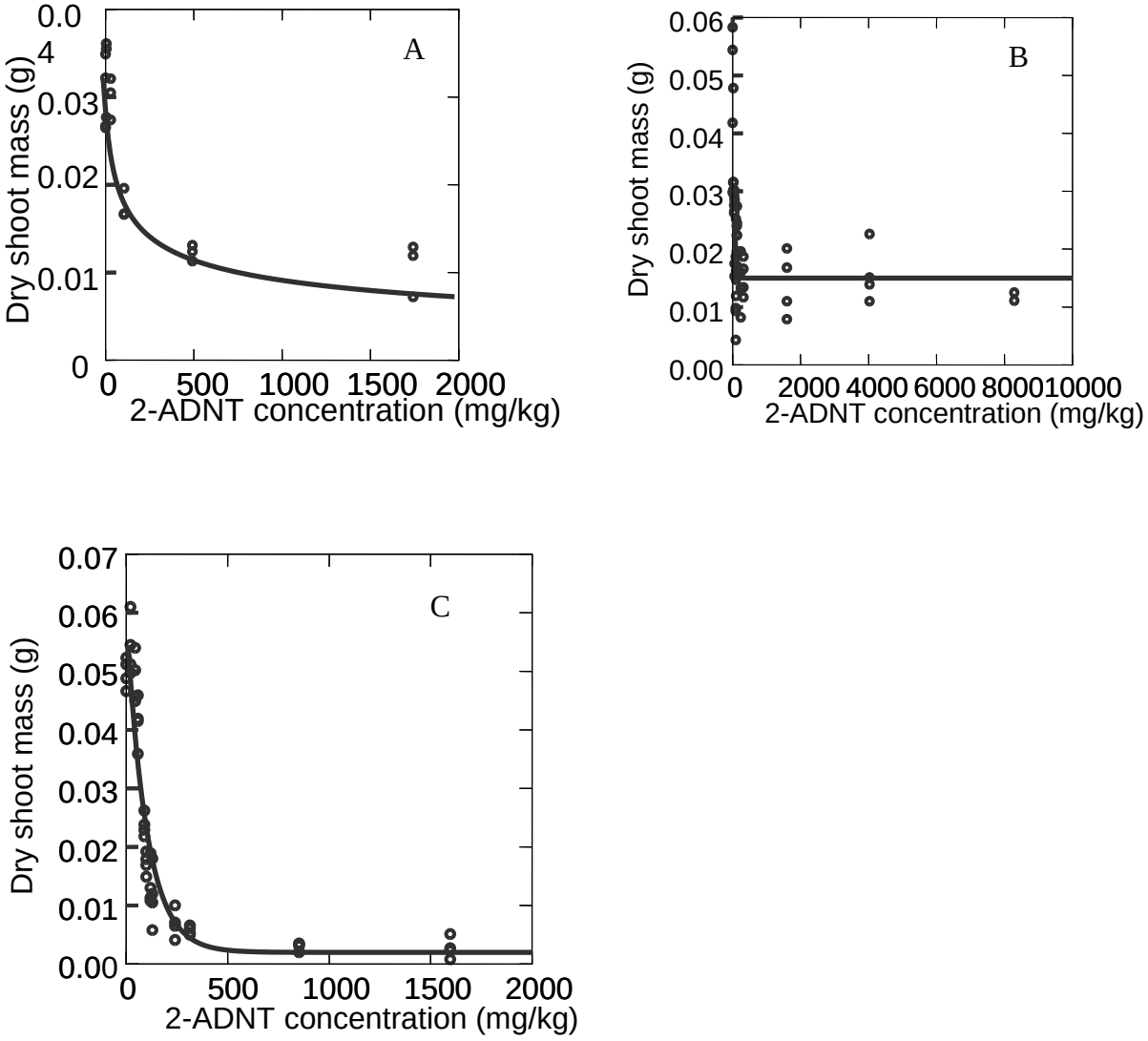


Figure 14. Effects of 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth determined in the definitive test.

5.4. Phytotoxicity of 4-ADNT

5.4.1. Effects of 4-ADNT on terrestrial plants in freshly amended SSL soil

Nominal concentrations selected for these tests were 0 (negative control), 0' (carrier control), 10, 20, 60, 180, 600, 2000, and 10,000 mg/kg. Corresponding analytically determined concentrations of 4-ADNT in SSL2007d soil are presented in Table 15. Definitive toxicity tests were performed using four replicates of each soil treatment for each of three test species. Seedling emergence of alfalfa, barnyard grass, and ryegrass in the carrier control was 86, 81, and 91 percent, respectively, which complies with the quality control criterion for this endpoint.

The EC_{20} values for freshly amended 4-ADNT (and corresponding 95 percent CI, mg/kg, for shoot growth dry mass were 5 (1-8), 28 (19-36), and 33 (21-45) for alfalfa, barnyard grass and ryegrass, respectively (Table 32). The exponential model had the best fit for seedling emergence or shoot growth for each of the three species (Figure 15).

Table 32. Summary of toxicological parameters for 4-ADNT (mg/kg) freshly amended into Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	1711	147	45
p	<0.001	0.148	0.772
LOEC	8690	516	147
p	0.091	<0.001	0.007
EC_{20}	<3048	160	<256
Confidence interval (95%)	ND	95-225	ND
Model used (EC_{20})	ND	Exponential	ND
R^2	ND	0.983	ND
EC_{50}	3048	498	256
Confidence interval (95%)	0-9989	295-700	112-400
Model used	Exponential	Exponential	Exponential
R^2	0.989	0.983	0.995
Growth - Fresh mass			
NOEC	<7	13	45
p	1.000	0.521	0.287
LOEC	7	45	147
p or P(T<=t) two-tail	0.025	0.002	<0.001
EC_{20}	10	26	33
Confidence interval (95%)	6-14	19-33	20-46
EC_{50}	30	80	103
Confidence interval (95%)	17-43	58-101	62-143
Model used	Exponential	Exponential	Exponential
R^2	0.895	0.981	0.975
Growth - Dry mass			
NOEC	13	13	45
p	0.087	0.893	0.858
LOEC	45	45	147
p or P(T<=t) two-tail	<0.001	0.004	<0.001
EC_{20}	5	28	33
Confidence interval (95%)	1-8	19-36	21-45
EC_{50}	14	86	102
Confidence interval (95%)	3-26	60-111	65-139
Model used	Exponential	Exponential	Exponential
R^2	0.986	0.981	0.981

Table notes: Concentrations are based on USEPA Method 8330A; ND=Not Determined – could not be determined within the concentration range tested; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

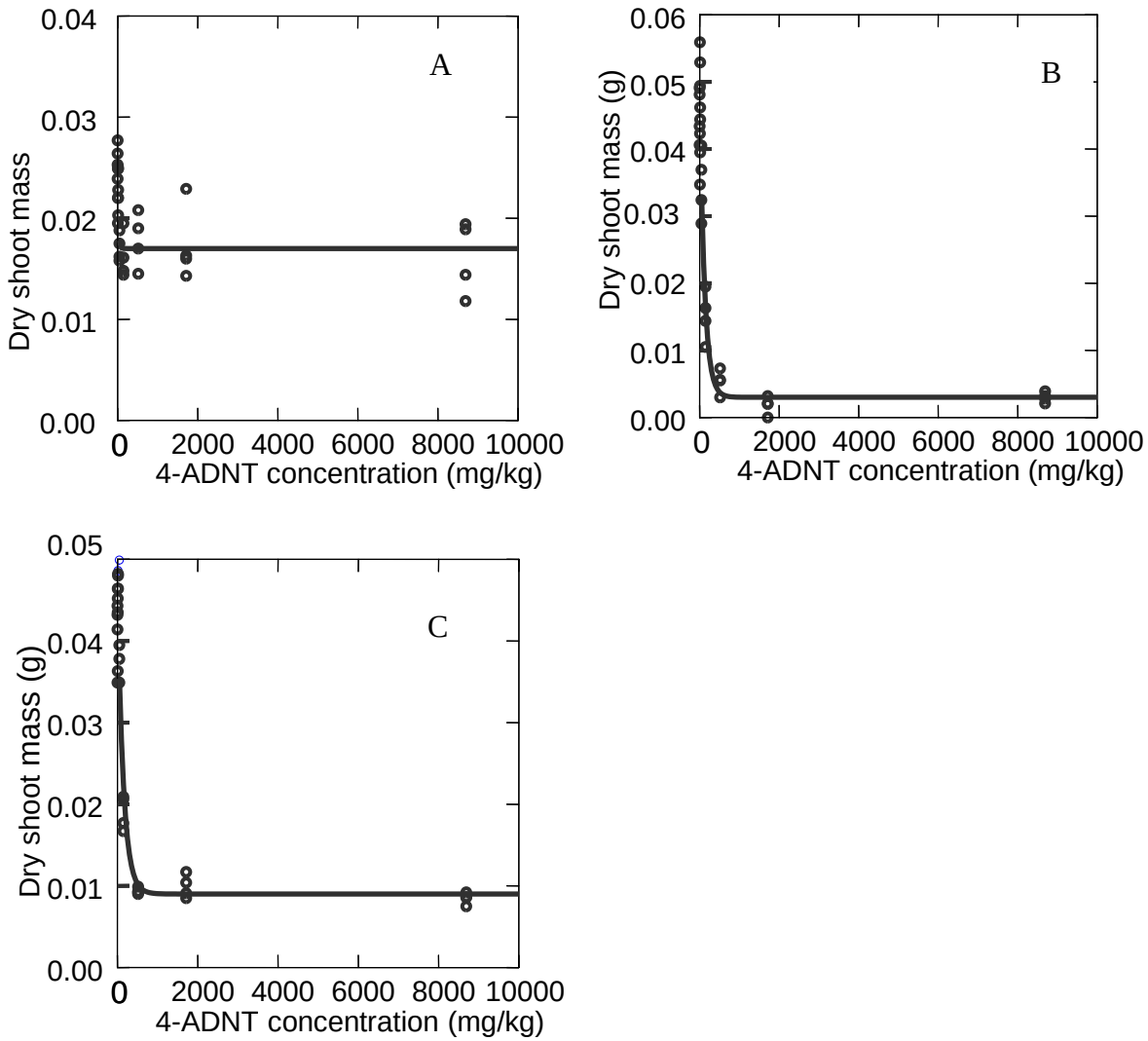


Figure 15. Effects of 4-ADNT on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth in freshly amended Sassafras sandy loam (SSL2007d) soil.

5.4.2. Effects of 4-ADNT weathered-and-aged in SSL soil on terrestrial plants

This study was designed to test the hypothesis that weathering-and-aging 4-ADNT in SSL soil can affect its toxicity to terrestrial plants. Nominal positive exposure concentrations of 4-ADNT in soil for the terrestrial definitive toxicity test were 40, 80, 160, 300, 400, 2000, 5000, and 10,000 mg/kg for alfalfa and barnyard grass; and 40, 80, 100, 160, 200, 400, 1000, and 2000 mg/kg for perennial ryegrass. Water and carrier (acetone) controls were included in these phytotoxicity tests. Corresponding analytically determined concentrations of 4-ADNT weathered-and-aged in SSL2007d soil are presented in Table 14. Tests were performed using

four replicates of each soil treatment for each of the three test species. 4-ADNT was weathered-and-aged in SSL2007d soil for 3 months. Seedling emergence of alfalfa, barnyard grass, and ryegrass in the carrier control was 84, 71, and 94 percent, respectively, which complies with the quality control criterion for this endpoint.

The exponential model had the best fit for both seedling emergence and shoot growth (fresh or dry mass) for all three test species (Figure 16). Values for regression coefficients determined for all EC_p endpoints were equal to or greater than 0.95, indicating a good fit of the model used for toxicity data. The EC_{20} values (and corresponding 95 percent CI), mg/kg, for shoot growth dry mass were 11 (0.1-23), 21 (7-34), and 130 (73-188) for alfalfa, barnyard grass and ryegrass, respectively (Table 33). Based on these EC_{20} results, the toxicity to ryegrass of 4-ADNT weathered-and-aged (EC_{20}= 130; CI= 73-188 mg/kg) in SSL2007d soil was significantly different from that of freshly amended 4-ADNT (EC_{20}= 33; CI= 21-45 mg/kg). No significant difference due to weathering-and-aging of 4-ADNT in soil was observed for alfalfa or barnyard grass.

Table 33. Summary of toxicological parameters for 4-ADNT (mg/kg) weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	8634	3277	724
p	0.194	0.742	0.196
LOEC	>8634	8634	3277
p	ND	0.019	<0.001
EC_{20}	<188	7620	<1772
Confidence intervals (95%)	ND	4326-10915	ND
Model used	ND	Gompertz	ND
R^2	ND	0.978	ND
EC_{50}	188	>7620	1772
Confidence intervals (95%)	0-790	ND	0-3696
Model used	Exponential	ND	Exponential
R^2	0.990	ND	0.997
Growth - Fresh mass			
NOEC	22	22	243
p	0.760	0.677	0.528
LOEC	63	63	724
p or P(T<=t) two-tail	<0.001	0.025	<0.001
EC_{20}	15	21	127
Confidence intervals (95%)	7-23	8-33	78-176
EC_{50}	46	65	395
Confidence intervals (95%)	22-70	25-104	242-548
Model used	Exponential	Exponential	Exponential
R^2	0.979	0.909	0.977
Growth - Dry mass			
NOEC	22	22	243
p	0.877	0.698	0.441
LOEC	63	63	724
p or P(T<=t) two-tail	0.017	0.061	<0.001
EC_{20}	11	21	130
Confidence intervals (95%)	0.1-23	7-34	73-188
EC_{50}	35	64	404
Confidence intervals (95%)	0.4-70	22-106	226-583
Model used	Exponential	Exponential	Exponential
R^2	0.972	0.921	0.976

Table notes: Concentrations are based on USEPA Method 8330A; ND=Not Determined: could not be determined within the concentration range tested; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

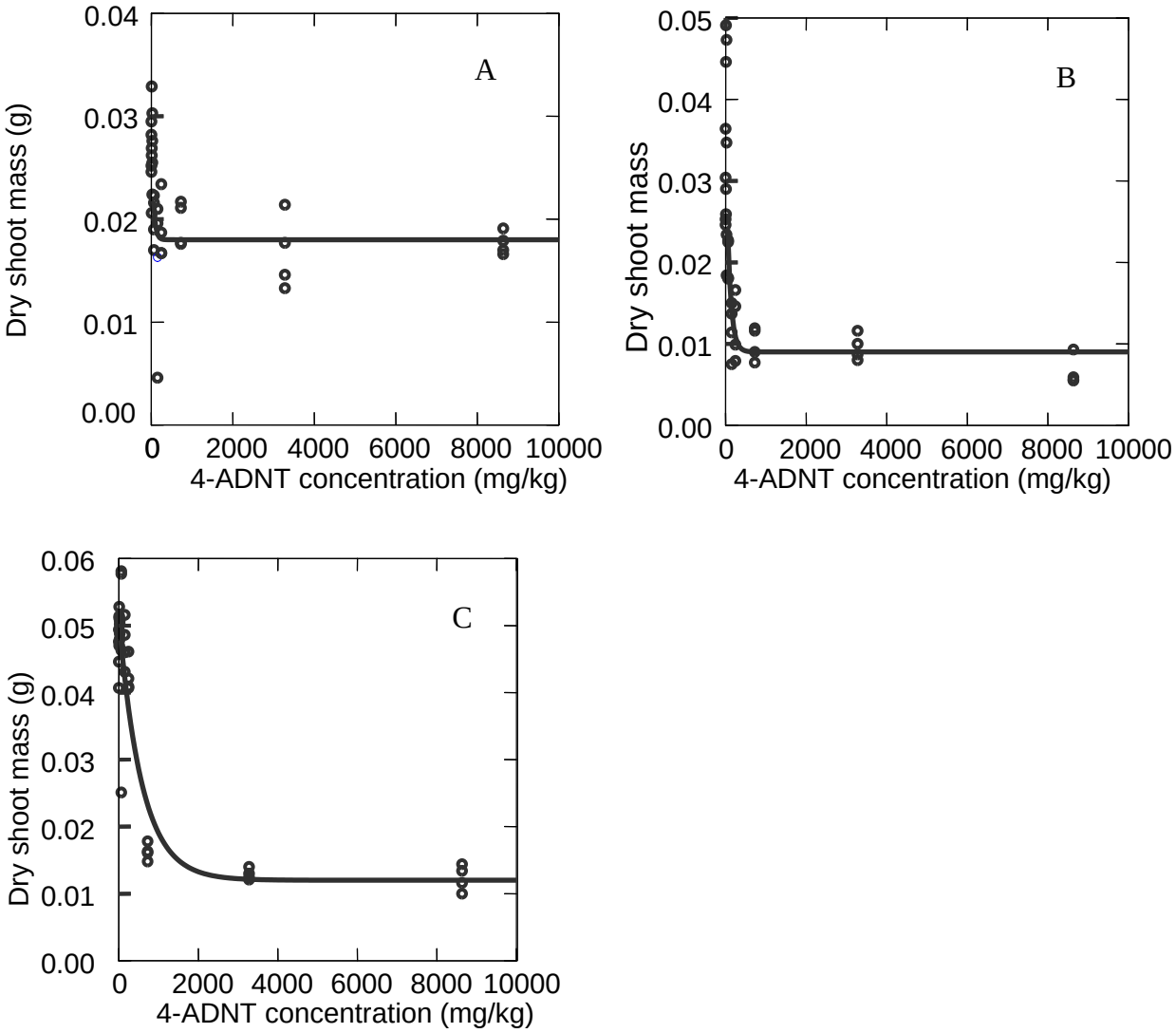


Figure 16. Effects of 4-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth.

5.5. Phytotoxicity of HMX

Based on the results of the soil invertebrate or terrestrial plant toxicity testing in SSL soil (SERDP project CU-1221), a composite toxicity test design with variable replication was used in this investigation. This design combined a range-finding test component and a definitive limit test component. The range-finding test component included the selection of a limited number of treatment concentrations and a reduced number of replicates for the intermediate treatments in

order to determine concentration range for the definitive study. The Limit Test component included increased replication in the carrier (acetone) control, and in the greatest treatment concentration (10000 mg/kg). The Limit Test is a variant of the definitive test, and is performed when statistical analysis of sufficient range-finding test data shows no significant effect at all treatment levels. Such composite toxicity test design expedites assessment by utilizing Limit Test results when no adverse effects are found, yet provides necessary information for a definitive test when the effect of exposure on measurement endpoints is statistically significant using the range-finding component. This composite toxicity test design was exceptionally useful and beneficial for determining the effects of HMX on potworms, Collembola, and plants.

Based on the results of the terrestrial plant toxicity testing in SSL soil, a composite range-finding/Limit Test was conducted to determine the effects of HMX weathered-and-aged in TSL for each of the three plant species, alfalfa, barnyard grass, and ryegrass. Nominal concentrations of HMX in soil for these tests were 0 (negative control), 0’ (carrier control), 100, 1000, 5000, and 10000 mg/kg. Corresponding analytically determined concentrations of HMX in TSL2002 soil are presented in Table 17. The following replication was used: four replicates in the 100, 1000, and 5000 mg/kg treatments, and eight replicates in the 0 (negative control), 0’ (acetone control), and 10000 mg/kg treatments. Seedling emergence of alfalfa, barnyard grass, and ryegrass in respective carrier controls was 80, 83, and 90 percent, which complies with the quality control criterion for this endpoint.

Results showed no statistically significant (p>0.05) adverse effects of HMX weathered-and-aged in TSL soil on seedling emergence or shoot growth (Table 34). The only significant effect measured was stimulation of alfalfa shoot growth, based on a 21% increase for shoot fresh mass and 45% increase for shoot dry mass (Figure 17).

Table 34. Summary of toxicological parameters for HMX (mg/kg) weathered-and-aged in Teller sandy loam (TSL2002) soil determined in toxicity tests with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	10208	10208	10208
P	0.468	0.569	0.822
LOEC	>10208	>10208	>10208
p	0.468	0.569	0.822
Growth - Fresh mass			
NOEC	4888	10208	10208
P	0.757	0.430	0.836
LOEC	10208	>10208	>10208
p	0.016	0.430	0.836
Growth - Dry mass			
NOEC	4888	10208	10208
P	0.119	0.640	0.270
LOEC	10208	>10208	>10208
p	<0.001	0.640	0.270

Table notes: Concentrations are based on USEPA Method 8330A; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

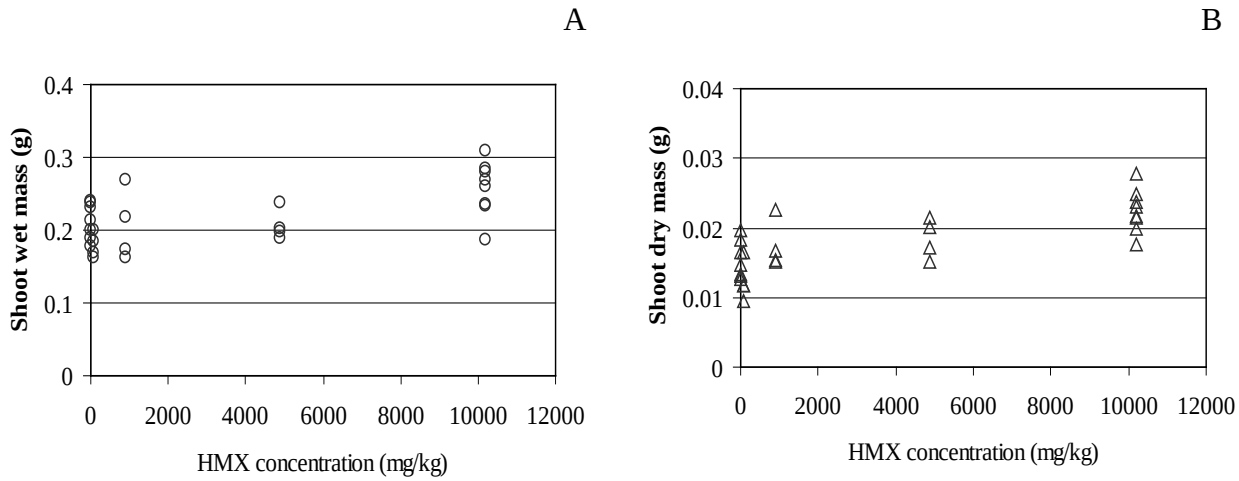


Figure 17.Effects of HMX weathered-and-aged in Teller sandy loam soil on alfalfa shoot fresh mass (A) and shoot dry mass (B).

5.6. Phytotoxicity of NG

5.6.1. Effects of NG on terrestrial plants in freshly amended SSL soil – range-finding toxicity tests

Range-finding plant toxicity tests were done in triplicate with NG freshly amended into SSL2004 soil. Nominal positive exposure concentrations of NG in soil were 1, 10, 100, 1000, and 5000 mg/kg, for alfalfa, barnyard grass, and perennial ryegrass. Water and carrier (acetone) controls were included in these phytotoxicity tests. Corresponding analytically determined concentrations of NG freshly amended into SSL2007d soil are presented in Table 19. Tests were performed using three replicates of each soil treatment for each of the three test species. Seedling emergence of alfalfa, barnyard grass, and ryegrass in the carrier control was 92, 82, and 88 percent, respectively, which complies with the quality control criterion for this endpoint.

The effects of NG on alfalfa, barnyard grass, and ryegrass are presented in Figures 18, 19, and 20. Results indicate that NG was lethal to all three plants at concentrations greater than 898 mg NG/kg dry SSL soil. Shoot growth was a more sensitive measurement endpoint for NG phytotoxicity than seedling emergence. Because 20% and 50% reductions of the measurement endpoints were obtained at concentrations between 0.8 and 898 mg NG/kg, definitive plant toxicity tests were conducted within this range of NG concentrations.

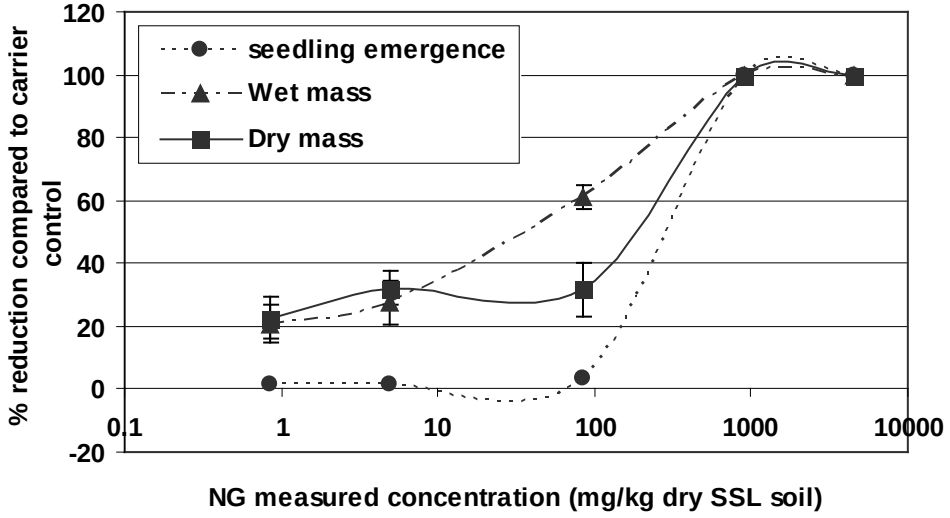


Figure 18. Effect of NG on alfalfa seedling emergence and shoot growth in freshly amended SSL soil.

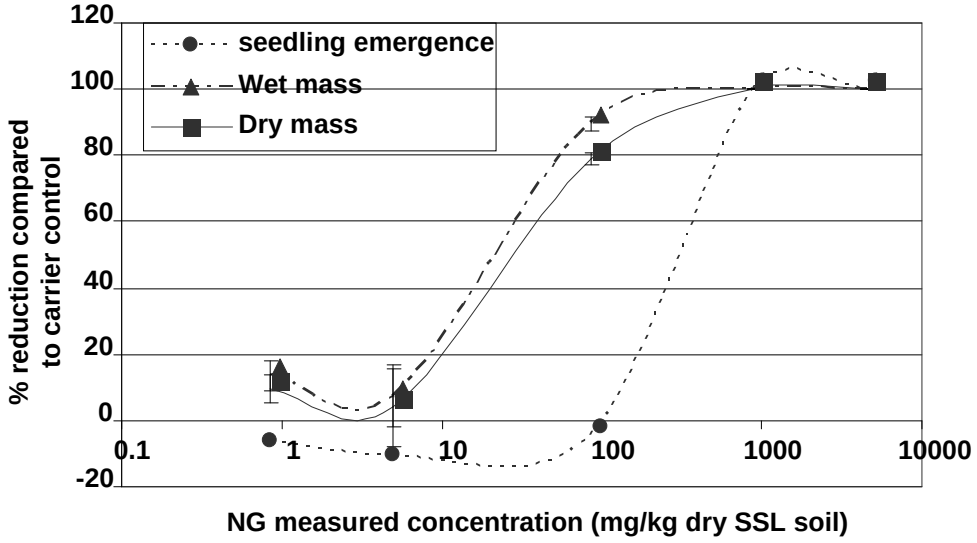


Figure 19. Effect of NG on barnyard grass seedling emergence and shoot growth in freshly amended into SSL soil.

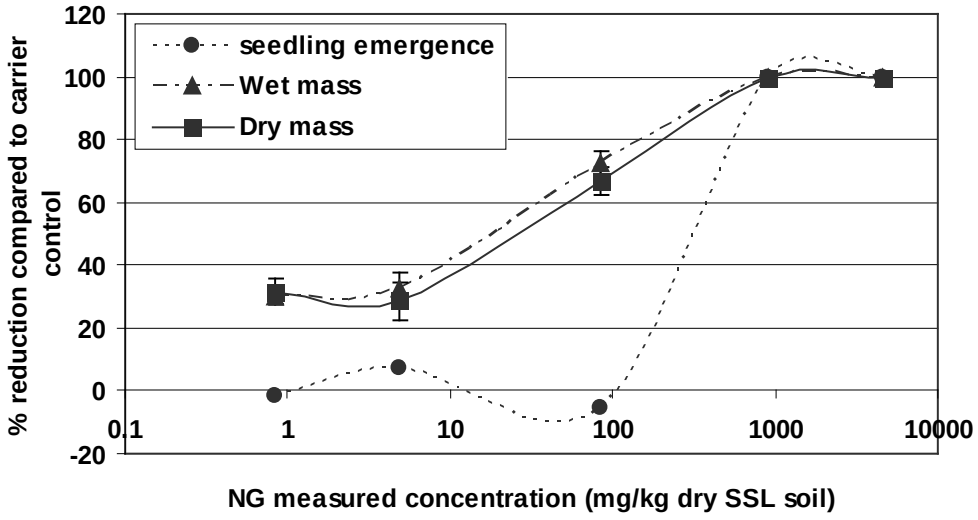


Figure 20. Effect of NG on ryegrass seedling emergence and shoot growth in freshly amended SSL soil.

The exponential model had the best fit for either seedling emergence or shoot growth (fresh or dry mass) data (Figure 21). Values for regression coefficients determined for all ECp endpoints were equal to or greater than 0.967, indicating a good fit of the model used for these toxicity data. EC_{20} values (and corresponding 95 percent CI), mg/kg, for shoot growth dry mass were 74 (13-136), 13 (9-16), and 20 (9-31), for alfalfa, barnyard grass, and ryegrass, respectively (Table 35). These results indicated that barnyard grass and ryegrass, both monocotyledonous species, were more sensitive to NG than the dicotyledonous species alfalfa.

Table 35. Summary of toxicological parameters for NG (mg/kg) freshly amended into Sassafras sandy loam soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Seedling emergence			
NOEC	85	0.8	85 [††]
p	0.454	0.143	0.205
LOEC	898	5	898 [‡]
p	<0.001	0.023	<0.001
EC_{20}	95	97	105
Confidence interval	50-140	50-144	43-167
EC_{50}	296	301	325
Confidence interval	157-435	156-447	135-515
Model used (EC_{20})	Exponential	Exponential	Exponential
R^2 (EC_{20})	0.989	0.988	0.981
Growth - Fresh mass			
NOEC	<0.8 [†]	5	<0.8 [†]
p	<0.001	0.088	<0.001
LOEC	0.8	85	0.8
p or P(T<=t) two-tail	<0.001	<0.001	<0.001
EC_{20}	23	9	16
Confidence interval	14-32	6-13	7-25
EC_{50}	71	29	50
Confidence interval	43-100	19-40	23-78
Model used (EC_{20})	Exponential	Exponential	Exponential
R^2 (EC_{20})	0.982	0.992	0.967
Growth - Dry mass			
NOEC	<0.84 [†]	5	<0.8 [†]
p	0.001	0.378	<0.001
LOEC	0.84	85	0.8
p or P(T<=t) two-tail	<0.001	<0.001	<0.001
EC_{20}	74	13	20
Confidence interval	13-136	9-16	9-31
EC_{50}	231	40	62
Confidence interval	40-421	29-50	29-95
Model used (EC_{20})	Exponential	Exponential	Exponential
R^2 (EC_{20})	0.973	0.993	0.968

Table notes: Concentrations are based on USEPA Method 8330A; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration; NOAEC=no-observed-adverse-effect concentration; LOAEC=lowest-observed-adverse-effect concentration. [†]Unbounded NOEC; [††]NOAEC; [‡]LOAEC.

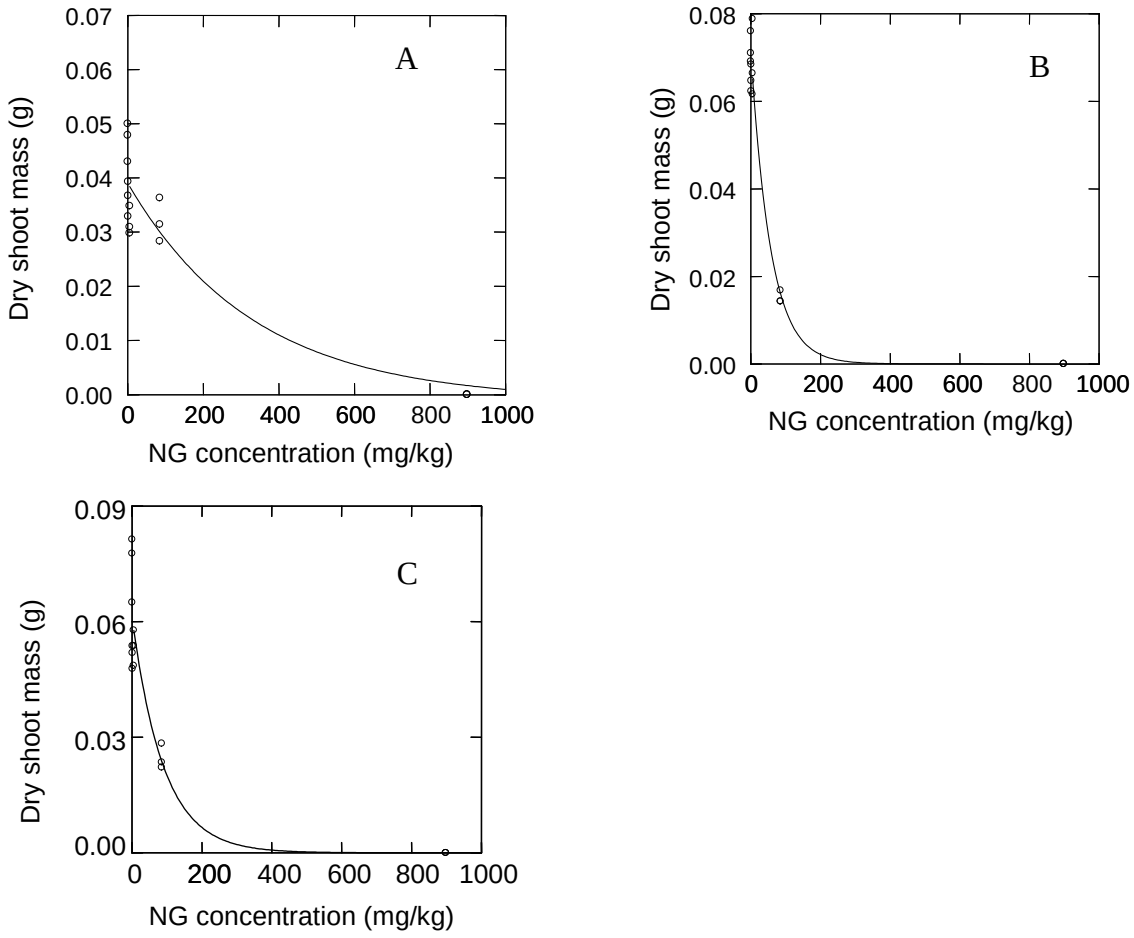


Figure 21. Effects of NG on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth in freshly amended Sassafras sandy loam (SSL2007d) soil.

5.6.2. Effects of NG weathered-and-aged in SSL soil on terrestrial plants

Nominal positive exposure concentrations of NG in soil for the terrestrial definitive toxicity test were 2, 5, 10, 20, 50, 100, 200, 400, and 650 mg/kg for alfalfa, barnyard grass, and perennial ryegrass. Water and carrier (acetone) controls were included in these phytotoxicity tests. Corresponding analytically determined concentrations of NG weathered-and-aged in SSL2007d soil are presented in Table 21. Tests were performed using four replicates of each soil treatment for each of the three test species. NG was weathered-and-aged in SSL2007d soil for 1 month. Seedling emergence of alfalfa, barnyard grass, and ryegrass, respectively, in carrier controls was 83, 69, and 89 percent. In order to improve the barnyard grass seedling emergence in the SSL2007d, which was below the 80% required by the quality control criterion for this endpoint, soil hydration was decreased to 60% of the SSL soil WHC (instead of 75% of the WHC). Using 60% of the WHC conditions, seedling emergence of barnyard grass was 76%, which complied with the quality control criterion for this endpoint.

The logistic (Gompertz) model had the best fit for either seedling emergence or shoot growth (fresh or dry mass) data (Figure 22). Values for regression coefficients determined for all ECp endpoints were greater than 0.96, indicating a good fit of the model used for toxicity data. The EC_{20} values (and corresponding 95 percent CI), mg/kg, for shoot growth dry mass were 83 (25-141), 12 (1-23), and 26 (12-41) for alfalfa, barnyard grass, and ryegrass, respectively (Table 36). These results indicated that barnyard grass and ryegrass, both monocotyledonous species, were more sensitive to NG than alfalfa, which is a dicotyledonous species.

Table 36. Summary of toxicological parameters for NG (mg/kg) weathered-and-aged in Sassafras sandy loam soil determined in the definitive toxicity test with alfalfa, barnyard grass, and ryegrass.

Ecotoxicological parameters	Alfalfa	Barnyard grass	Ryegrass
Soil hydration (% of the WHC)	75	60	75
Seedling emergence			
NOEC	122	33	21
p	0.530	0.140	0.953
LOEC	268	126	122
p	0.011	<0.001	<0.001
EC_{20}	286	56	97
Confidence intervals (95%)	175-398	29-83	50-144
EC_{50}	485	126	250
Confidence intervals (95%)	0-1132	96-157	195-306
Model used	Gompertz	Gompertz	Gompertz
R^2	0.988	0.983	0.989
Growth - Fresh mass			
NOEC	1.8	7	21
p	0.875	0.894	0.383
LOEC	21	33	122
p or P(T<=t) two-tail	<0.001	<0.001	<0.001
EC_{20}	5	16	42
Confidence intervals (95%)	0-13	0-38	9-75
EC_{50}	77	24	73
Confidence intervals (95%)	29-125	9-39	43-103
Model used	Gompertz	Gompertz	Gompertz
R^2	0.977	0.967	0.982
Growth - Dry mass			
NOEC	21	7	1.8
p	0.347	0.468	0.299
LOEC	122	33	21
p or P(T<=t) two-tail	0.005	<0.001	0.020
EC_{20}	83	12	26
Confidence intervals (95%)	25-141	1-23	12-41
EC_{50}	185	23	63
Confidence intervals (95%)	131-238	14-32	44-81
Model used	Gompertz	Gompertz	Gompertz
R^2	0.969	0.967	0.986

Table notes: Concentrations are based on USEPA Method 8330A; WHC=Water Holding Capacity; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

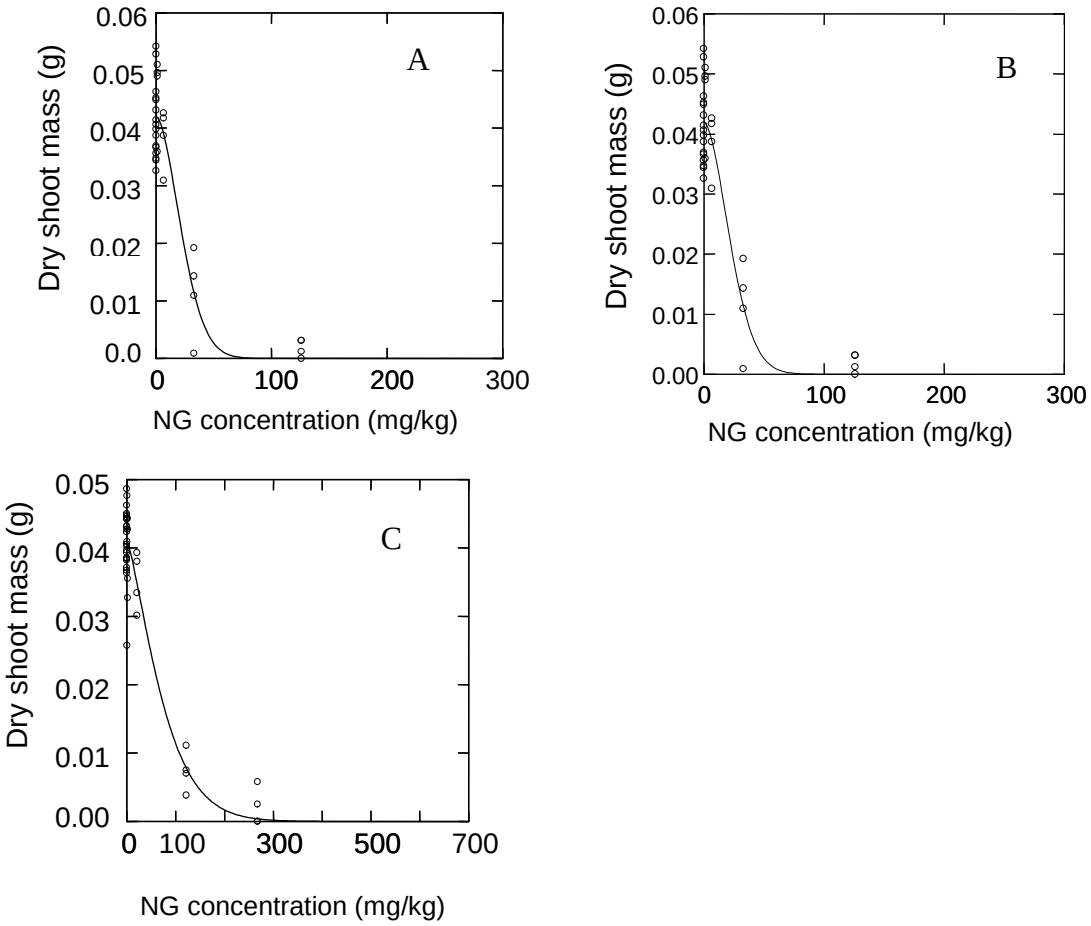


Figure 22. Effects of NG weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on alfalfa (A), barnyard grass (B), and ryegrass (C) shoot growth.

5.7. Discussion: Effects of energetic materials on terrestrial plants

This portion of the project was undertaken to produce scientifically-defensible toxicity data for the development of terrestrial plants-based Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG, and to investigate and characterize predominant soil physico-chemical parameters that can affect the bioavailability and resulting toxicity of 2,4-DNT to terrestrial plants. To achieve the first objective, studies were designed to meet specific USEPA criteria (USEPA, 2005). Eco-SSL test acceptance criteria were met or exceeded in these investigations by ensuring that: (1) tests were conducted in natural soils having physico-chemical characteristics that support high relative bioavailability of the EM compounds tested; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species was specified and appropriate.

Definitive toxicity studies using terrestrial plants exposures in TSL or SSL soil established new ecotoxicological data for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG under conditions of very high relative bioavailability for organic chemicals in soil (as defined in USEPA, 2005). The present studies confirmed the toxicity benchmarks established for 2,4-DNT in our previous studies with SSL soil (Rocheleau *et al.,* 2006; 2010). Toxicological benchmarks established in the present studies for 2,4-DNT (shoot growth EC_{20} values ranging between 5 to 120 mg/kg) and for HMX (LOEC = 10,208 mg/kg) were also consistent with data reported in the comprehensive review by Kuperman *et al.* (2009). In addition, the present studies confirmed that shoot growth was a more sensitive endpoint than seedling emergence, which is a generally accepted paradigm (Natr and Lawlor, 2005). Finally, we observed that the sensitivity of the three studied plant species (alfalfa, barnyard grass, and ryegrass) was dependent on the EM and the type of soil used.

Our hypothesis that weathering-and-aging 2-ADNT, 4-ADNT, or NG in SSL soil would affect its toxicity to terrestrial plants was invalidated because there was no significant difference between the toxicities of freshly amended and weathered-and-aged EMs.These results contrast with findings of our previous studies which demonstrated that the weathering-and-aging of other nitroaromatic energetic compounds in soil significantly increased their respective toxicities, including that of 2,4,6-trinitroluene (TNT), 1,3,5-trinitrobenzene (TNB), and dinitrotoluenes (Rocheleau *et al*., 2006; 2008). This difference may be explained, in part, by a relatively high biodegradability of NG for example, compared to the less easily degraded nitroaromatic compounds, such as TNT (Podlipná *et al*., 2008).

Our second objective was to characterize predominant soil physico-chemical parameters that can affect the bioavailability and resulting toxicity of 2,4-DNT to terrestrial plants. Based on the shoot growth endpoint, 2,4-DNT was more toxic in TSL soil, followed by KCL soil, and WCL soil. Organic matter content of the soil was strongly and significantly correlated with shoot growth toxicity benchmarks for 2,4-DNT weathered-and-aged in soil. Soil clay content explained between 45 and 61 percent in variance of shoot growth toxicity benchmarks for 2,4-DNT, however, none of the correlations were statistically significant. These results identified soil organic matter as the dominant property mitigating 2,4-DNT toxicity for the three plant species tested in the present studies. A more extensive discussion of these results is presented in Section 6.7.

6. Toxicity of energetic materials on soil invertebrates

6.1. Toxicity of boric acid

6.1.1. Toxicity of boric acid to earthworms

Earthworm 56-day Reproduction Toxicity Tests with boric acid were conducted periodically to serve as positive controls in order to evaluate integrity of the earthworm cultures throughout the study period. Results of these studies are presented in the form of Warning Chart values completed 2 March 2005, 4 January 2006, 7 March2007, 10 October 2007, and 3 June 2008, using the SSL2007d soil. Benchmarks for boric acid were determined by nonlinear regression analyses of toxicity data for *E. fetida*. The test completed in March 2007 yielded the respective EC_{50} values and corresponding 95% CL of 845 (831-859) and 160 (101-219) mg/kg for adult survival and juvenile production, respectively. The test completed in October 2007 yielded the EC_{50} values and corresponding 95% CL of 1385 (1127-1642) and 140 (30-249) mg/kg for adult survival and juvenile production, respectively. These values were plotted on a Boric Acid Warning Chart to monitor the condition of the earthworm culture (Figures 23 and 24). The values were within both the Warning Limits and 95% CL established for *E. fetida* culture in previous tests with boric acid. This confirmed that the current condition of *E. fetida* culture met the validity requirements of the test protocol.

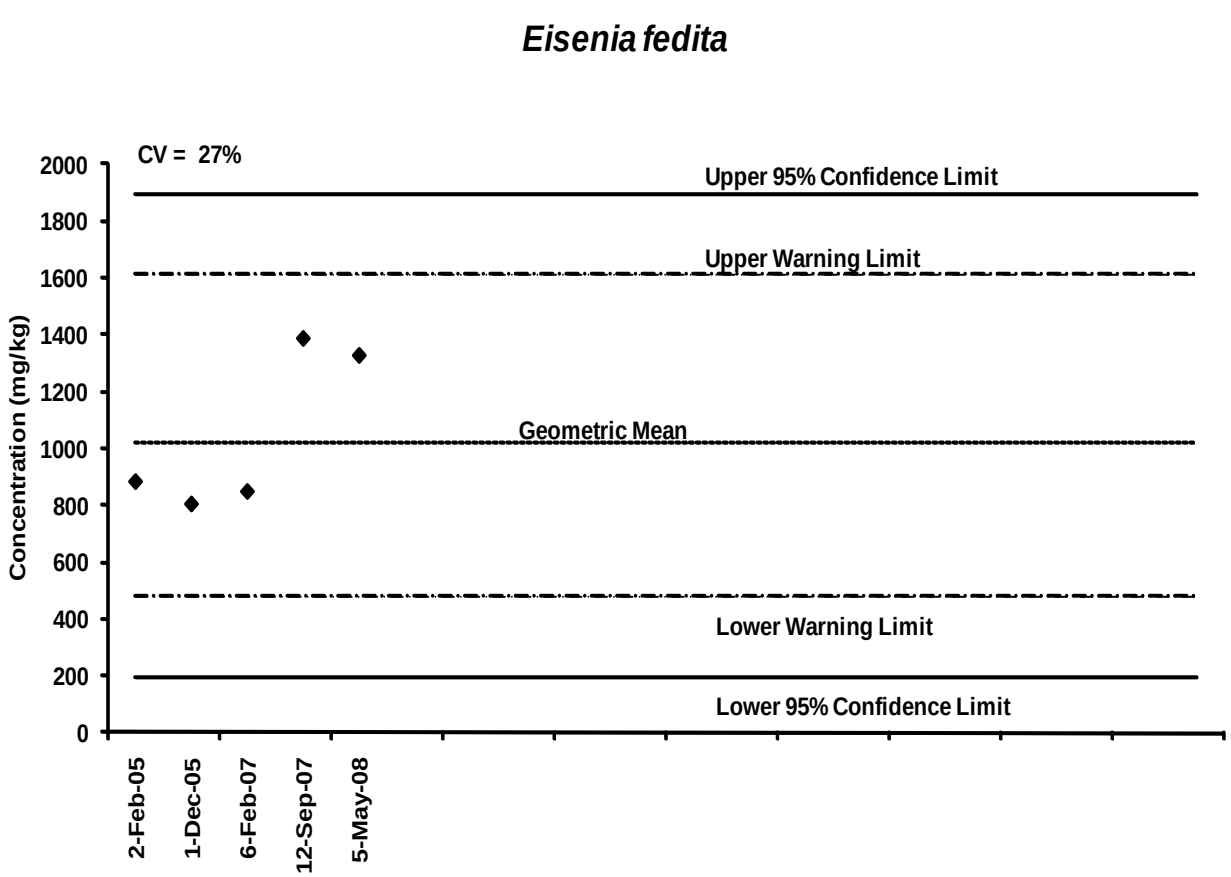


Figure 23.Warning Chart for the *Eisenia fetida* culture showing the 28-d EC_{50} values for adult survival established in the definitive tests with the reference toxicant, boric acid in Sassafras sandy loam soil.

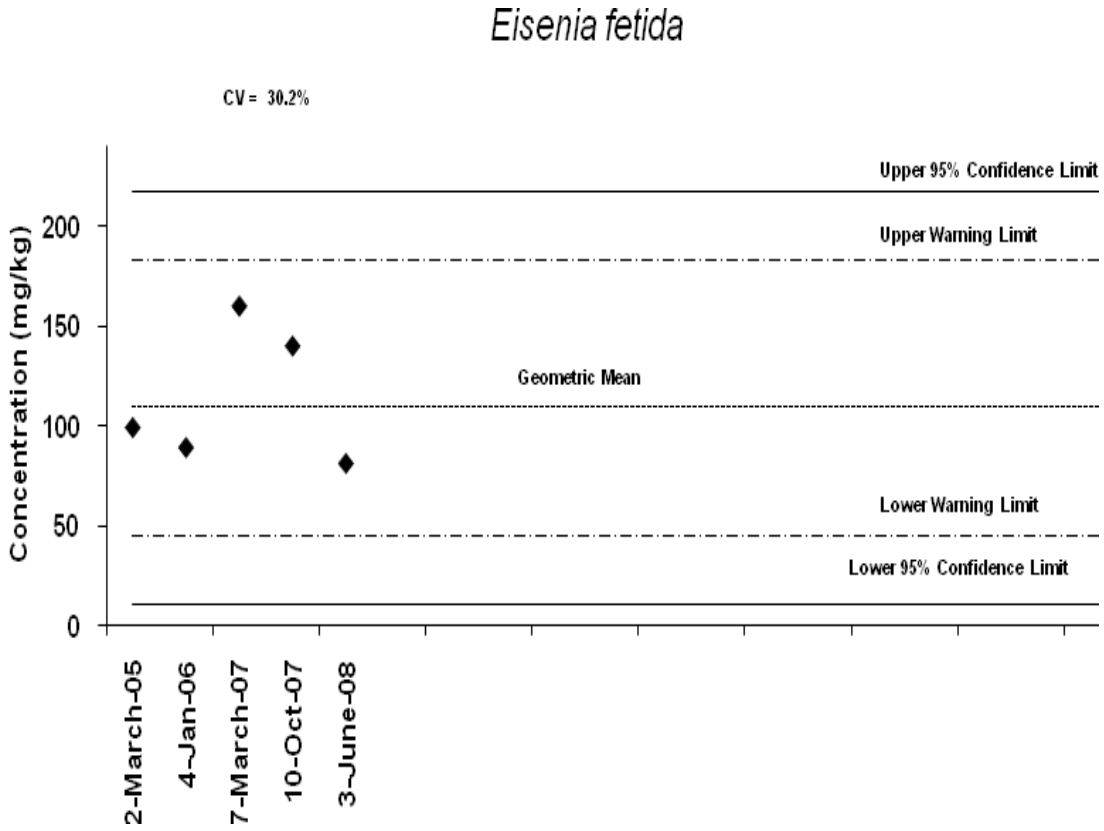


Figure 24. Warning Chart for the *Eisenia fetida* culture showing the 56-d EC_{50} values for juvenile production established in the definitive tests with the reference toxicant, boric acid in Sassafras sandy loam soil.

6.1.2. Toxicity of boric acid to potworms

Toxicity tests with reference toxicant boric acid (positive control) were conducted throughout the project to assess changes in sensitivity, health and performance of *E. crypticus* maintained in ECBC laboratory cultures. Nonlinear regression analyses of toxicity data from independent studies with SSL soil were used to establish the respective EC_{50} values and corresponding 95% CL for juvenile production. All determined EC_{50} values were within both the Warning Limits of ± 2 standard deviations and 95% CL established for *E. crypticus* culture in tests with boric acid (Figure 25). The coefficient of variation (CV) was 8.4% (less than CV of 20% suggested as reasonable by EC, 2005). These results confirmed that the condition of *E. crypticus* culture met the validity requirements of the test protocol.

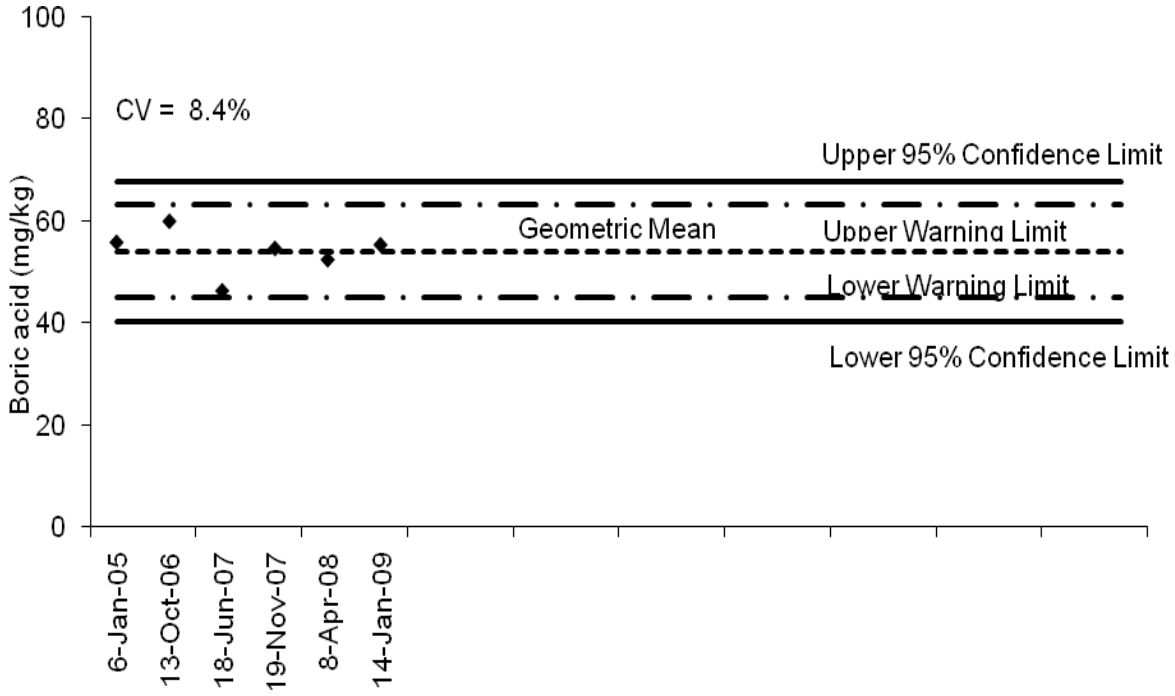


Figure 25. Warning Chart for the *Enchytraeus crypticus* culture showing the EC_{50} values for juvenile production established in the definitive tests with the reference toxicant, boric acid in Sassafras sandy loam soil.

6.1.3. Toxicity of boric acid to Collembola

Toxicity tests with reference toxicant boric acid (positive control) were conducted throughout the project using the SSL soil to assess changes in sensitivity, health and performance of *F. candida* maintained in ECBC laboratory cultures. Nonlinear regression analyses of toxicity data from independent studies were used to establish the respective EC_{50} values and corresponding 95% CL for juvenile production. All determined EC_{50} values were within the Warning Limits of ± 2 standard deviations and 95% CL established for *F. candida* culture in tests with boric acid (Figure 26). The coefficient of variation (CV) was 8.4% (less than CV of 20% suggested as reasonable by EC, 2005). These results confirmed that the condition of *F. candida* culture met the validity requirements of the test protocol.

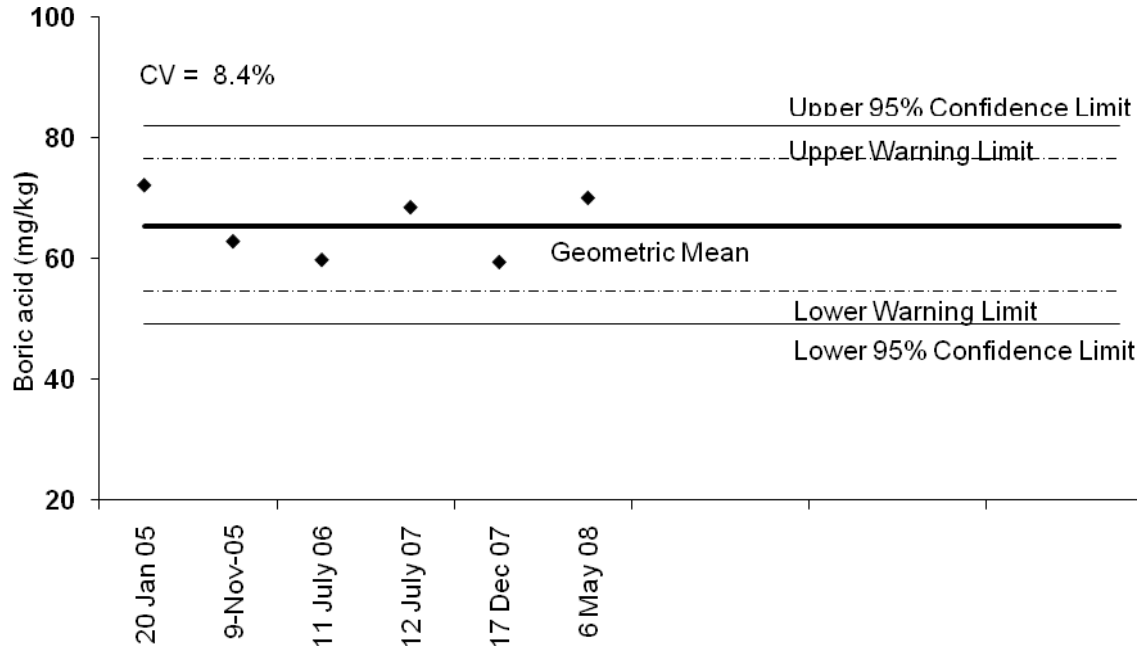


Figure 26. Warning Chart for the *Folsomia candida* culture showing the EC_{50} values for juvenile production established in the definitive tests with the reference toxicant, boric acid in Sassafras sandy loam soil.

6.2. Toxicity of 2,4-DNT

6.2.1. Effects of 2,4-DNT weathered-and-aged in soil on the earthworms

Definitive 56-day reproduction toxicity tests were performed to determine the toxicity of 2,4-DNT weathered-and-aged in each of the TSL, KL, and WCL soils (corresponding soil batch designations were TSL2002, KL2006, and WCL2001) to the earthworm, *E. fetida*. Separate toxicity tests were performed using each of the three soil types. The current standard Earthworm Reproduction Test is ISO 11268-2 (ISO, 1998a). Nominal treatment concentrations of 2,4-DNT used in the toxicity studies were 2, 5, 10, 20, 40, 60, 80, 100, 200, and 400 mg/kg in TSL, WCL, and KL soils (Tables 5, 7, and 8). An additional nominal concentration of 600 mg/kg was used in the test with KL soil. All soil treatments were brought to 95% of the respective soil WHC (13, 20, and 23% of the TSL, KL, and WCL soil dry mass, respectively), 24 h prior to commencement of earthworm toxicity tests.

Validity criteria were met for data produced in the Earthworm Reproduction tests, done in each of the three soils. The validity criteria for the negative controls for this test are as follows: Coefficient of Variation (CV) ≤ 30% and adult survival ≥ 90% in negative controls. In the

present studies, the CV in the negative controls was 17, 22, and 20% in tests conducted with TSL, WCL, and KL soils, respectively. Adult survival in the negative controls was 100% in tests conducted in all three soils.

Both the number of cocoons and the number of juveniles produced by earthworms in all three soil types were significantly reduced ($p < 0.05$; compared with carrier control treatments) when *E. fetida* were exposed to 2,4-DNT that was weathered-and-aged in soil. The NOEC and LOEC values are shown in Table 37. For cocoon production, the bounded NOEC/LOEC values mg/kg (i.e., mg 2,4-DNT/kg dry soil mass, DM) for TSL, KL, and WCL soils were 15/29, 36/51, and 65/115, respectively (Table 37). The bounded NOEC/LOEC values for juvenile production in TSL, KL, and WCL soils were 15/29, 3/5, and 65/115, respectively (Table 37). The bounded NOEC/LOEC values for adults in TSL or KL soils were 67/160 and 51/115, respectively (Table 37). Adult survival was not affected in WCL soil containing 2,4-DNT concentrations ≤346 mg/kg DM (Table 37).

Data from earthworm reproduction tests were fit to nonlinear regression models. Reproduction data from tests with TSL soils and KL soils fit best into the logistic Gompertz model (Figures 27 and 28). Reproduction data from tests with WCL soil fit best into the logistic Hormetic model (Figure 29). The EC_{20}/EC_{50} values (mg/kg) derived from the regression analyses for production of cocoons were 20/36, 32/55, and 81/107 in TSL, KL, and WCL soil, respectively (Table 37). For production of juveniles, the EC_{20}/EC_{50} values were 5/14, 2/14, and 67/88 mg/kg in TSL, KL, and WCL soil, respectively (Table 37). In these studies, 2,4-DNT was significantly (95% CI basis) more toxic to cocoon and juvenile production by *E. fetida* in TSL or KL soils than in WCL soil (Table 37). Toxicity was not significantly different (95% CI basis) between TSL and KL soils. In KL soil, EC_{20} and EC_{50} values indicated that 2,4-DNT was significantly (95% CI basis) more toxic to juvenile production than to cocoon production (Table 37). The EC_{20} and EC_{50} values for juvenile production were also lower than for cocoon production in TSL and WCL soils, although the differences were not statistically significant (Table 37).

Table 37. Summary of toxicological benchmarks determined in the definitive tests with *Eisenia fetida* for 2,4-DNT weathered-and-aged in Teller sandy loam (TSL), Kirkland loam (KL), and Webster clay loam (WCL) soils.

Ecotoxicological parameter	2,4-DNT (mg/kg)	2,4-DNT (mg/kg)	2,4-DNT (mg/kg)
Soil type	TSL	KL	WCL
Adult survival			
NOEC	67	51	≥346[a]
p	0.21	1.0	1.0
LOEC	160	115	>346[a]
p	0.001	0.001	1.0
Cocoon production			
NOEC	15	36	65
p	0.57	0.14	0.923
LOEC	29	51	115
p	0.003	0.006	0.001
EC_{20}	20	32	81
Confidence intervals (95%)	8-32	16-41	67-96
EC_{50}	36	55	107
Confidence intervals (95%)	26-46	37-72	85-128
Model used	Gompertz	Gompertz	Hormetic
R^2	0.90	0.93	0.96
Juvenile production			
NOEC	15	3	65
p	0.18	0.07	0.221
LOEC	29	5	115
p	0.013	0.001	0.001
EC_{20}	5	2	67
Confidence intervals (95%)	0-11	0-4	50-84
EC_{50}	14	14	88
Confidence intervals (95%)	4-24	4-25	57-119
Model used	Gompertz	Gompertz	Hormetic
R^2	0.86	0.91	0.89

Table notes: Values are soil concentration means determined by USEPA Method 8330A. EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. NOEC and LOEC values were derived from Analysis of Variance procedures and FLSD pairwise means comparison test. [a]Adults were not significantly reduced ($p>0.05$) up to and including 346 mg/kg in WCL soil. The ECp values were derived from nonlinear regression models (see Figures 27-29).

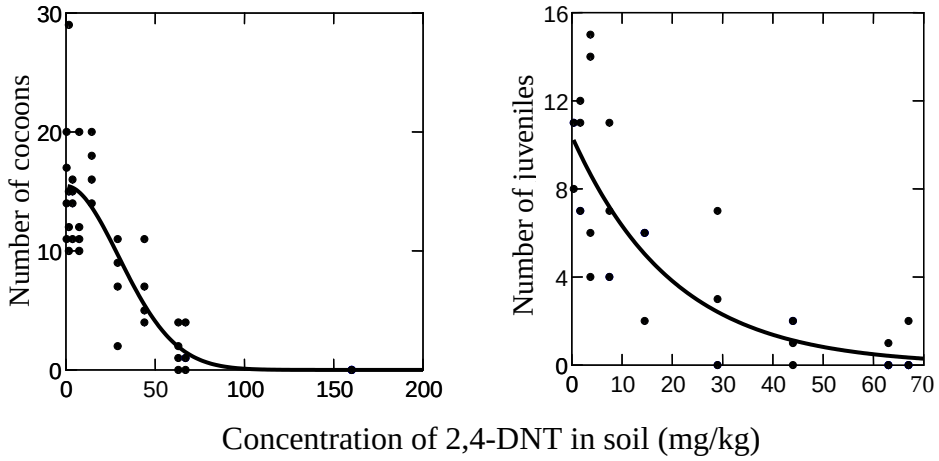


Figure 27. Nonlinear regression (logistic Gompertz model) of *Eisenia fetida* reproduction data and 2,4-DNT concentrations in Teller sandy loam soil.

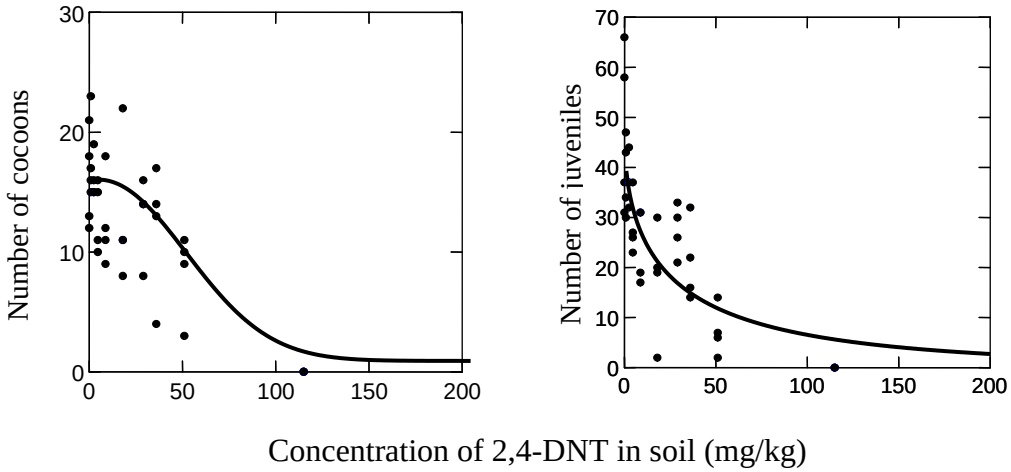


Figure 28. Nonlinear regression (logistic Gompertz model) of *Eisenia fetida* reproduction data and 2,4-DNT concentrations in Kirkland loam soil.

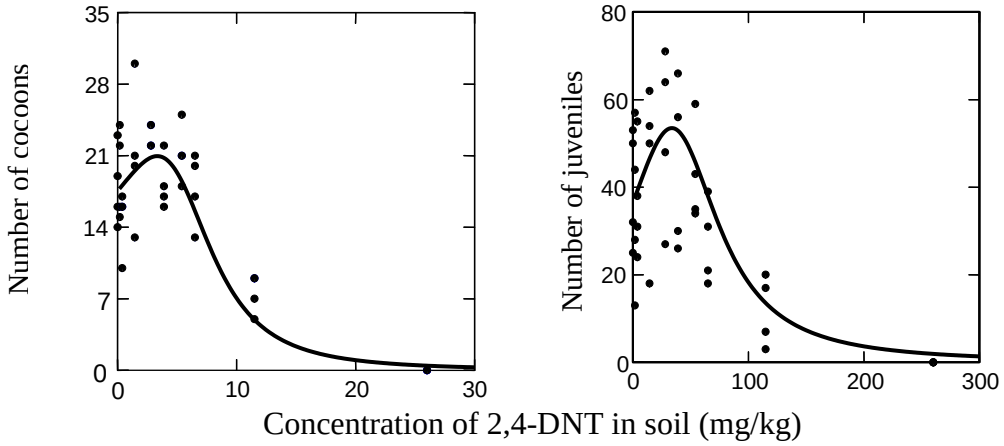


Figure 29. Nonlinear regression (logistic Hormetic model) of *Eisenia fetida* reproduction data and 2,4-DNT concentrations in Webster clay loam soil.

The relationships among the 2,4-DNT toxicity benchmarks for *E. fetida* and the soil properties were determined using Pearson's correlation analysis. Toxicity data for *E. fetida* established in our previous similarly designed studies with Sassafras sandy loam (SSL2000) soil (SERDP CU-1221) were included in this analysis. All linear correlations were performed on the original (untransformed) data. Pearson's linear correlation coefficients (r) and their respective probability (p) values are summarized in Table 38.

Table 38. Pearson correlation coefficients (r) for key soil properties and 2,4-DNT toxicity benchmarks for adult survival and reproduction endpoints determined in definitive tests with *Eisenia fetida*.

Toxicity benchmark	Clay %	p	OM %	p	pH	p	CEC cmol/kg	p
Adult Survival								
EC_{20}	0.949	0.051	0.980	0.020	0.703	0.297	0.960	0.040
EC_{50}	0.950	0.050	0.992	0.008	0.683	0.317	0.943	0.057
Cocoons								
EC_{20}	0.971	0.029	0.988	0.012	0.726	0.274	0.946	0.054
EC_{50}	0.978	0.022	0.975	0.025	0.762	0.238	0.969	0.031
Juveniles								
EC_{20}	0.904	0.097	0.916	0.084	0.640	0.360	0.776	0.224
EC_{50}	0.963	0.037	0.955	0.045	0.729	0.271	0.876	0.124

Table notes: Pearson correlation coefficients with corresponding probabilities (p) were determined using data from the definitive toxicity tests with Sassafras sandy loam, Teller sandy loam, Kirkland loam, and Webster clay loam soils. Estimates of effect concentration (EC) producing a 20% (EC_{20}) or 50% (EC_{50}) decrease in the measurement endpoint compared with acetone control were determined for 2,4-DNT weathered-and-aged in soil. OM = organic matter content of the soil; CEC= cation exchange capacity of the soil.

Organic matter content of the soil was strongly ($r \geq 0.975$) and significantly ($p \leq 0.025$) correlated with all adult survival and cocoon production toxicity benchmarks for 2,4-DNT. Juvenile production EC_{50} was significantly correlated ($r = 0.955$; $p = 0.045$) with OM, but EC_{20} was not ($r = 0.916$; $p = 0.084$) (Table 38). Soil clay content was strongly ($r \geq 0.904$) correlated with all toxicity benchmarks for 2,4-DNT. These correlations were statistically significantly ($p \leq 0.05$) for adult survival EC_{50}, and cocoon production EC_{20} and EC_{50} benchmarks (Table 38). Strong correlations were also detected for adult survival and cocoon production toxicity benchmarks and soil CEC ($r \geq 0.943$), with statistical significance for adult survival EC_{50} and cocoon production EC_{50} (Table 38), which are likely a result of significant collinearity between clay and CEC found in soils used in these studies. No significant ($p \geq 0.238$) correlations were found among any toxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter and clay as the dominant properties mitigating 2,4-DNT toxicity to *E. fetida*.

6.2.2. Effects of 2,4-DNT on the potworm Enchytraeus crypticus

Definitive toxicity tests were performed separately and independently for 2,4-DNT freshly amended into soils, and for 2,4-DNT weathered-and-aged in TSL, KL, and WCL soils (corresponding soil batch designations were TSL2002, KL2006, and WCL2001), in order to

determine toxicity benchmark values for 2,4-DNT in each exposure and soil type. Toxicity data for 2,4-DNT established in our studies with SSL soil (corresponding soil batch designation SSL2000) were reported previously in Kuperman *et al.* (2006b) and are included in Tables 39 and 40 of this report. Treatment concentrations of 2,4-DNT in each soil were prepared as single batches for toxicity studies (Tables 5, 7, and 8). Each batch was analyzed to determine 2,4-DNT concentration at the time of introducing the test species. Following treatment batch preparation (described earlier in this report), 100-g samples of freshly amended soil were collected from each selected soil treatment batch and stored at -80°C for three months prior to toxicity testing. Definitive toxicity testing with the freshly amended TSL, KL, and WCL soils were conducted at approximately the same time (within one week) as testing of 2,4-DNT weathered-and-aged in the respective soil types to minimize potential seasonal variability in reproduction rates of *E. crypticus*. The TSL, KL, and WCL soil samples used in definitive toxicity testing with 2,4-DNT, either freshly amended or weathered-and-aged in soil, were hydrated with ASTM type I water to 100% of the respective soil WHC (13, 20, and 23% of the TSL, KL, and WCL soil dry mass, respectively), and were allowed to equilibrate for 24 h before exposing the potworms.

Test results complied with the validity criteria defined in the ISO/16387 (2004) test guideline and those stipulated in Section 3.11.2 of this report. The respective validity criteria test results from a single negative control treatment used for concurrent studies of 2,4-DNT freshly amended into soil and for 2,4-DNT weathered-and-aged in soil, were for TSL: mean adult survival 98%, mean number of juveniles produced 334, coefficient of variation (CV) 24%; for KL: mean adult survival 100%, mean number of juveniles produced 1921, CV 6 %; and for WCL: mean adult survival 95%, mean number of juveniles produced 1857, CV 11%. Results of definitive tests with a reference toxicant, boric acid (positive control) are shown in Figure 26 and discussed in Section 4.3.1.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the 2,4-DNT treatments.

Ecotoxicological responses of *E. crypticus* to 2,4-DNT freshly amended and to 2,4-DNT weathered-and-aged in each soil are shown in Tables 39 and 40, respectively. Both adult survival and juvenile production were affected in 2,4-DNT-amended soils within the concentration ranges selected for definitive tests. Juvenile production was the more sensitive measurement endpoint for assessing 2,4-DNT toxicity to *E. crypticus* in all soil types tested, compared with adult survival, which comports with results of our previous studies. The logistic Gompertz model had the best fit for data in all toxicity tests, except for juvenile production data in test with 2,4-DNT weathered-and-aged in TSL, where the logistic hormetic model, having an additional parameter to accommodate hormesis (a stimulatory effect caused by exposure to low concentrations of a chemical followed by inhibitory effects at higher concentrations), had the best fit (Figures 30-35). Values for regression coefficients (R^2) determined for toxicity endpoints were ≥0.916 in tests with 2,4-DNT freshly amended into soils, and ≥0.977 in tests with 2,4-DNT weathered-and-aged in soils (Tables 39 and 40), indicating good fit of the models used for toxicity data.

Weathering-and-aging 2,4-DNT in sandy loam soils significantly (95% CI basis) decreased the toxicity to *E. crypticus* based on the EC_{20} values for juvenile production in TSL and the EC_{50} values for adult survival in SSL, compared to these effects levels in respective freshly amended soils. In contrast, weathering-and-aging 2,4-DNT in clay loam soils significantly (95% CI basis) increased the toxicity to *E. crypticus* based on the EC_{50} values for juvenile production in KL and the EC_{50} values for adult survival in WCL, compared to these effects levels in respective freshly amended soils (Tables 39 and 40).

Table 39. Summary of toxicological benchmarks (mg/kg) for 2,4-DNT freshly amended into Teller sandy loam (TSL), Sassafras sandy loam (SSL), Kirkland loam (KL), and Webster clay loam (WCL) soils determined in the definitive tests with *Enchytraeus crypticus*.

Ecotoxicological parameter	TSL	SSL[§]	KL	WCL
Adult survival				
NOEC	64	40.9	110	440
P	0.275	0.659	0.618	0.085
LOEC	84	55	160	677
P	0.004	0.013	<0.0001	<0.0001
EC_{20}	32	57	207	473
Confidence interval (95%)	0-83	54-60	194-221	443-503
EC_{50}	157	67	243	554
Confidence interval (95%)	42-272	64-70	230-257	529-579
Model used	Gompertz	Gompertz	Gompertz	Gompertz
R^2	0.942	0.994	0.992	0.996
Juvenile production				
NOEC	10.5	9.9	20	64
P	0.151	0.271	0.879	0.073
LOEC	15.4	20.3	42	80
P	0.010	0.037	0.022	0.004
EC_{20}	9	19	52	189
Confidence interval (95%)	1-17	13-26	45-60	139-239
EC_{50}	28	36	106	287
Confidence interval (95%)	15-40	30-41	98-113	249-325
Model used	Gompertz	Gompertz	Gompertz	Gompertz
R^2	0.916	0.980	0.994	0.967

Table notes: [§] Modified from Kuperman *et al.* (2006); Soil concentration of 2,4-DNT (mg/kg) were determined by USEPA Method 8330A; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

Table 40. Summary of toxicological benchmarks (mg/kg) for 2,4-DNT weathered-and-aged in Teller sandy loam (TSL), Sassafras sandy loam (SSL), Kirkland loam (KL), and Webster clay loam (WCL) soils determined in the definitive tests with *Enchytraeus crypticus*.

Ecotoxicological parameter	TSL	SSL[§]	KL	WCL
Adult survival				
NOEC	44	37	87	260
P	0.697	0.711	0.591	0.664
LOEC	63	72	115	447
P	0.010	0.015	<0.0001	<0.0001
EC_{20}	74	74*	184	404
Confidence interval (95%)	59-89	65-84	161-207	288-519
EC_{50}	112	101	203	467*
Confidence interval (95%)	102-123	62-140	173-233	410-524
Model used	Gompertz	Gompertz	Gompertz	Gompertz
R^2	0.992	0.994	0.972	0.994
Juvenile production				
NOEC	14.5	5.2	4.6	65
P	0.174	0.318	0.732	0.254
LOEC	29	11.8	8.7	97
P	<0.0001	<0.0001	0.009	<0.0001
EC_{20}	28*	14	38	122
Confidence interval (95%)	21-34	10-18	29-47	90-155
EC_{50}	41	27	80*	228
Confidence interval (95%)	35-46	24-31	70-89	198-258
Confidence interval (95%)				
Model used	Hormetic	Gompertz	Gompertz	Gompertz
R^2	0.977	0.983	0.987	0.984

Table notes: [§] Data from Kuperman *et al*. (2006); Soil concentration of 2,4-DNT (mg/kg) were determined by USEPA Method 8330A; *Statistically significant (95% confidence intervals basis) change in toxicity following weathering-and-aging of 2,4-DNT in soil; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

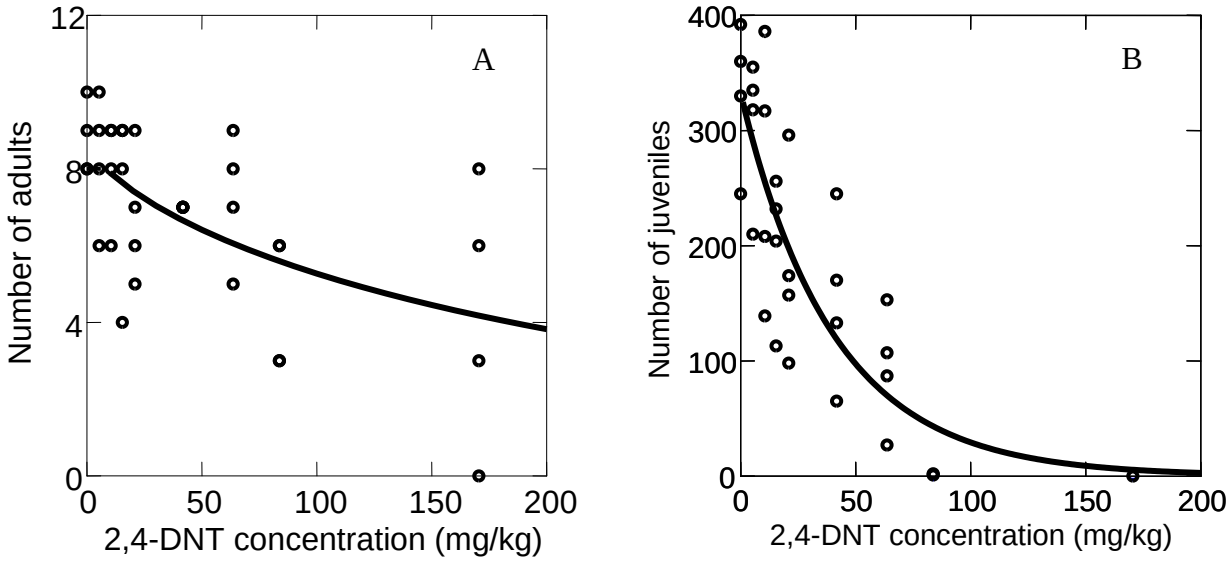


Figure 30. Effect of 2,4-DNT on adult survival (A) and juvenile production (B) by *Enchytraeus crypticus* in freshly amended Teller sandy loam.

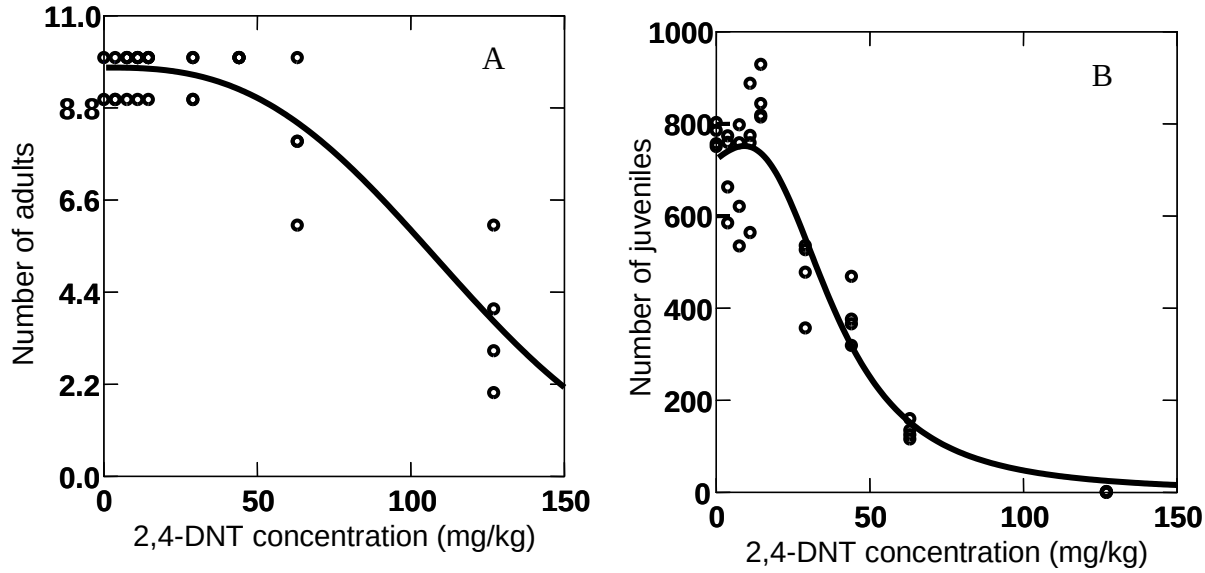


Figure 31. Effect of 2,4-DNT weathered-and-aged in Teller sandy loam on adult survival (A) and juvenile production (B) by *Enchytraeus crypticus.*

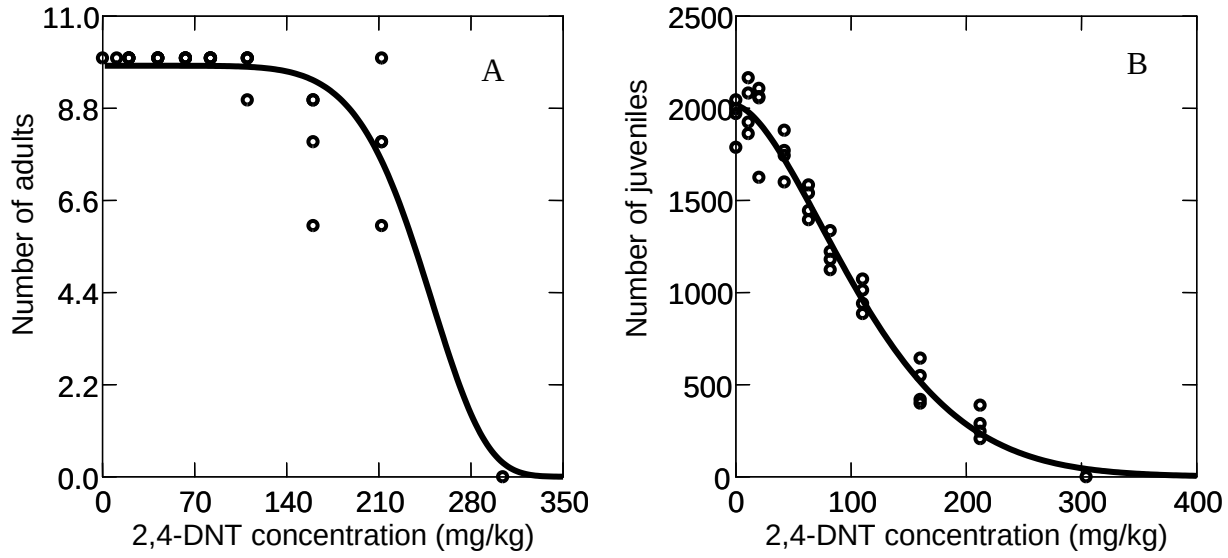


Figure 32. Effect of 2,4-DNT on adult survival (A) and juvenile production (B) by *Enchytraeus crypticus* in freshly amended Kirkland loam.

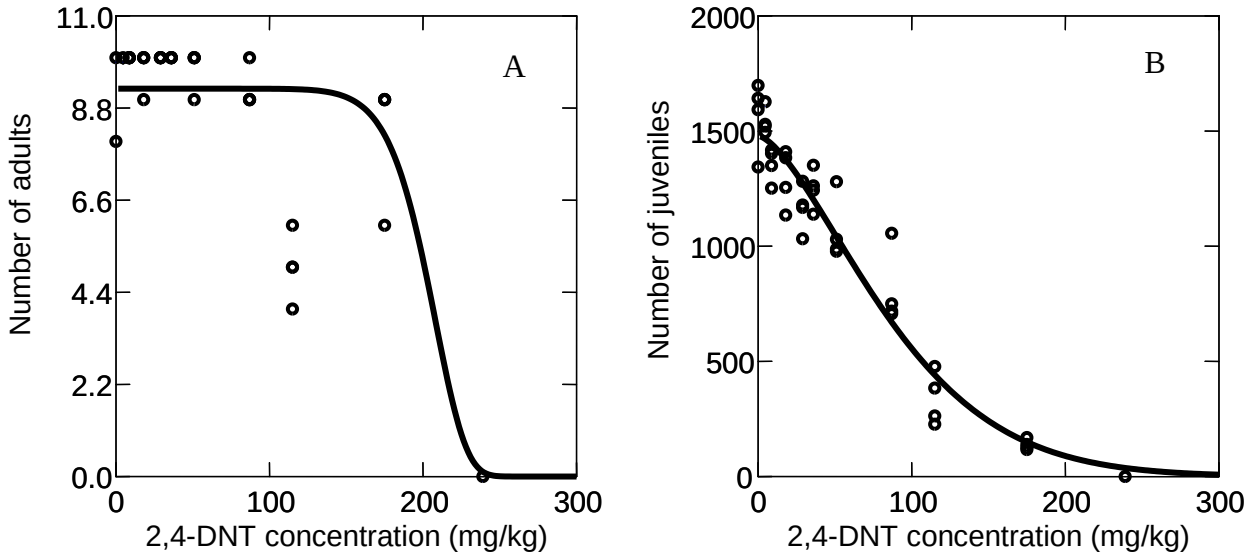


Figure 33. Effect of 2,4-DNT weathered-and-aged in Kirkland loam on adult survival (A) and juvenile production (B) by *Enchytraeus crypticus.*

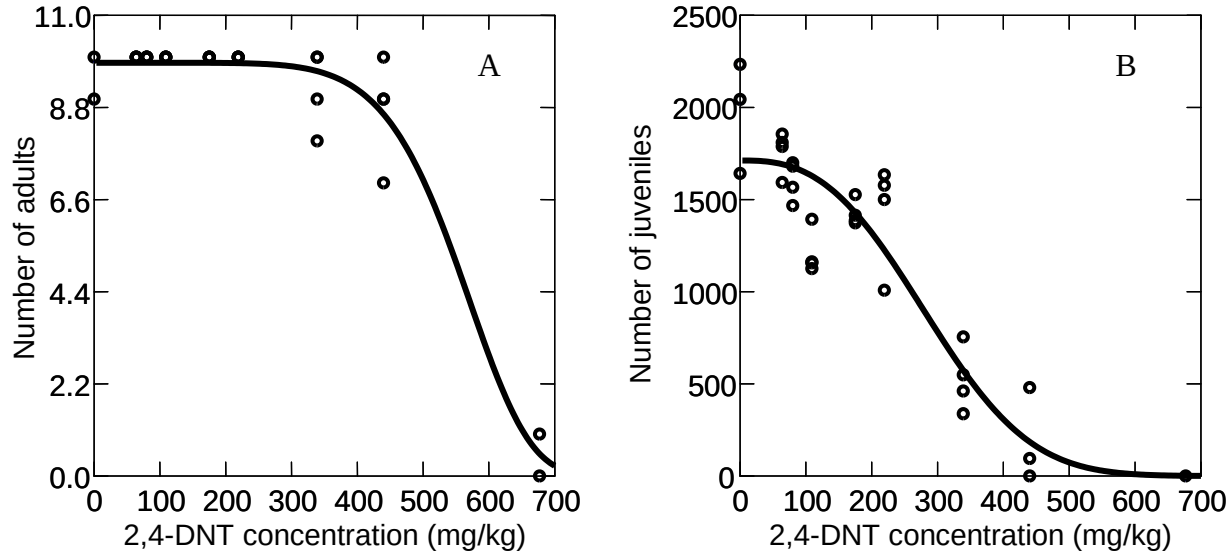


Figure 34. Effect of 2,4-DNT on adult survival (A) and juvenile production (B) by *Enchytraeus crypticus* in freshly amended Webster clay loam.

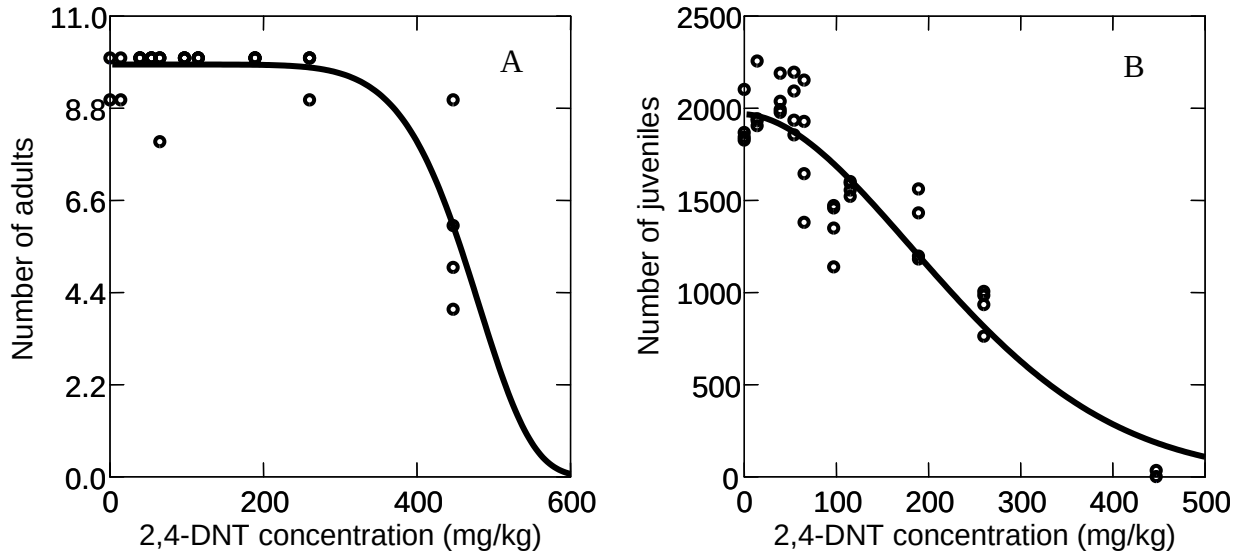


Figure 35. Effect of 2,4-DNT weathered-and-aged in Webster clay loam on adult survival (A) and juvenile production (B) by *Enchytraeus crypticus.*

The relationships among the 2,4-DNT toxicity benchmarks for *E. crypticus* and soil properties were determined using Pearson's correlation analysis. Toxicity data for *E. crypticus* established in our previous similarly designed studies with Sassafras sandy loam (SSL2000) soil (SERDP CU-1221) were included in this analysis. Soil-related differences were evident in adult survival

and reproduction (juvenile production) toxicity benchmarks for 2,4-DNT freshly amended or 2,4-DNT weathered-and-aged in each of the four natural soils tested in these studies. Reproduction toxicity (the main focus of these studies) to *E. crypticus* based on the EC_{50} values for 2,4-DNT freshly amended into soil was in the order (from greatest to least toxicity; smallest to greatest EC_{50} values): TSL > SSL > KL > WCL. The order for 2,4-DNT weathered-and-aged in soil was: SSL > TSL > KL > WCL. The effect of soil on 2,4-DNT toxicity was investigated by determining quantitative relationships between the concentration-response-based toxicity benchmark estimates (EC_{20} and EC_{50}) for juvenile production or adult survival endpoints and direct soil property measurements. All linear correlations were performed on the original (untransformed) data. Pearson's linear correlation coefficients (r) and their respective probability (p) values are summarized in Table 41. There was no statistically significant collinearity ($p = 0.076$) between soil organic matter and clay measurements, which are key soil constituents that could affect bioavailability of 2,4-DNT. There was significant correlation between clay content and soil CEC ($r = 0.960$, $p = 0.040$; data not shown).

Table 41. Pearson correlation coefficients (r) for key soil properties and 2,4-DNT toxicity benchmarks for adult survival and reproduction endpoints determined in the definitive tests with *Enchytraeus crypticus*.

Toxicity benchmark	Clay %	p	OM %	p	pH	p	CEC cmol/kg	p
Survival								
EC_{20}FA	0.975	0.025	0.940	0.060	0.648	0.352	0.992	0.008
EC_{50}FA	0.907	0.093	0.960	0.040	0.494	0.506	0.949	0.051
EC_{20}WA	0.966	0.034	0.958	0.042	0.596	0.404	0.982	0.018
EC_{50}WA	0.958	0.042	0.976	0.024	0.536	0.464	0.966	0.034
Reproduction								
EC_{20}FA	0.973	0.027	0.983	0.017	0.523	0.477	0.955	0.045
EC_{50}FA	0.973	0.027	0.969	0.031	0.573	0.427	0.974	0.026
EC_{20}WA	0.919	0.081	0.990	0.010	0.418	0.582	0.923	0.077
EC_{50}WA	0.946	0.054	0.982	0.018	0.497	0.503	0.953	0.047

Table notes: Pearson correlation coefficients with corresponding probabilities (p) were determined using data from the definitive toxicity tests with Sassafras sandy loam, Teller sandy loam, Kirkland loam, and Webster clay loam soils. Estimates of effect concentration (EC) producing a 20% (EC_{20}) or 50% (EC_{50}) decrease in the measurement endpoint compared with acetone control were determined for 2,4-DNT freshly amended (FA) in soil and for 2,4-DNT weathered-and-aged (WA) in soil. OM = organic matter content of the soil; CEC=cation exchange capacity of the soil.

Organic matter content of the soil was strongly ($r \geq 0.969$) and significantly ($p \leq 0.031$) correlated with all reproduction toxicity benchmarks for 2,4-DNT, and with adult survival benchmarks ($r \geq 0.958$; $p \leq 0.042$), except for the EC_{20} benchmark in freshly-amended soil

(Table 41). Soil clay content was strongly ($r \geq 0.907$) correlated with all toxicity benchmarks for 2,4-DNT. These correlations were statistically significantly ($p < 0.05$), except for adult survival EC_{50} benchmark in freshly-amended soil ($p = 0.093$), and the reproduction EC_{20} or EC_{50} benchmarks for 2,4-DNT weathered-and-aged in soil ($p \leq 0.081$). Strong correlations were also detected for toxicity benchmarks and soil CEC ($r \geq 0.923$) (Table 41), which are likely a result of significant collinearity between clay and CEC found in soils used in these studies. No significant ($p \geq 0.352$) correlations were found among any toxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter and clay as the dominant properties mitigating 2,4-DNT toxicity to *E. crypticus*.

6.2.3. Effects of 2,4-DNT weathered-and-aged in soil on Collembola

Definitive toxicity tests were performed separately and independently for 2,4-DNT weathered-and-aged in TSL, KL, and WCL soils (corresponding soil batch designations TSL2001, KL2006, and WCL2001), in order to determine toxicity benchmark values for 2,4-DNT in each soil type. Toxicity data for 2,4-DNT established in our previous study (SERDP CU-1221) with SSL soil (corresponding soil batch designation SSL2000) are included in Table 42 of this report. Treatment concentrations of 2,4-DNT in each soil were prepared as single batches for toxicity studies. Each batch was analyzed to determine 2,4-DNT concentration at the time of introducing the test species. The TSL, KL, and WCL soil samples used in definitive toxicity testing with 2,4-DNTwere hydrated with ASTM type I water to 88% of the respective soil WHC (13, 20, and 23% of the TSL, KL, and WCL soil dry mass, respectively), and were allowed to equilibrate for 24 h before exposing the collembolans.

Test results in TSL, KCL, and WCL complied with the validity criteria for the negative controls, as defined in the ISO/11267 test guideline and those stipulated in Section 3.11.3 of this report. The respective mean adult survival values for the carrier controls for TSL, KL, and WCL, were 88, 92, and 86%. The mean number of juveniles produced in the carrier controls were 176, 133, and 152 in TSL, KL, and WCL, respectively; with coefficients of variation equaling 7.7, 12.4 and 6.9% in TSL, KL, and WCL. Results of definitive tests with a reference toxicant, boric acid (positive control) are shown in Figure 26 and discussed in Section 4.3.1.3. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the 2,4-DNT treatments.

Ecotoxicological responses of *F. candida* to 2,4-DNT weathered-and-aged in each soil are shown in Table 42. Based on these results for *F. candida,* the reproduction toxicity for 2,4-DNT weathered-and-aged in four soils is in the order (from greatest toxicity to least) KL > SSL > TSL > WCL. Both adult survival and juvenile production were affected in 2,4-DNT-amended soils within the concentration ranges selected for definitive tests. Juvenile production was the more sensitive measurement endpoint for assessing 2,4-DNT toxicity to *F. candida* in all soil types tested, compared with adult survival, based on the respective EC_{20} or EC_{50} values. The logistic Gompertz model had the best fit for data in all toxicity tests (Figures 36-38). Values for regression coefficients (R^2) determined for toxicity endpoints were ≥0.954 (Table 42), indicating good fit of the models used for toxicity data.

Table 42. Summary of toxicological benchmarks (mg/kg) for 2,4-DNT weathered-and-aged in Teller sandy loam (TSL), Kirkland loam (KL), and Webster clay loam (WCL) soils determined in the definitive tests with *Folsomia candida*.

Ecotoxicological parameters	TSL	SSL[§]	KL	WCL
Adult survival				
NOEC	14.5	5.2	2.5	14.4
p	0.33	0.325	0.237	0.139
LOEC	29	11.5	4.6	28
p	<0.0001	<0.0001	0.022	0.029
EC_{20}	32	12	6	41
Confidence intervals (95%)	28-35	4-20	4-9	35-46
EC_{50}	38	38	14	60
Confidence intervals (95%)	36-40	27-48	11-17	54-65
Model used	Gompertz	Gompertz	Gompertz	Gompertz
R^2	0.986	0.983	0.969	0.987
Juvenile production				
NOEC	3.7	3	4.6	14.4
p	0.199	0.084	0.057	0.333
LOEC	7.5	5	8.7	28
p	0.039	0.001	<0.0001	<0.0001
EC_{20}	24	15	3	27
Confidence intervals (95%)	20-28	11-19	1-5	22-32
EC_{50}	30	23	10	47
Confidence interval (95%)	28-33	20-26	7-12	43-51
Model used	Gompertz	Gompertz	Gompertz	Gompertz
R^2	0.978	0.980	0.954	0.987

Table notes: Values are soil concentration means determined by USEPA Method 8330A. EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. [§] Benchmarks established in previous study (SERDP CU-1221).

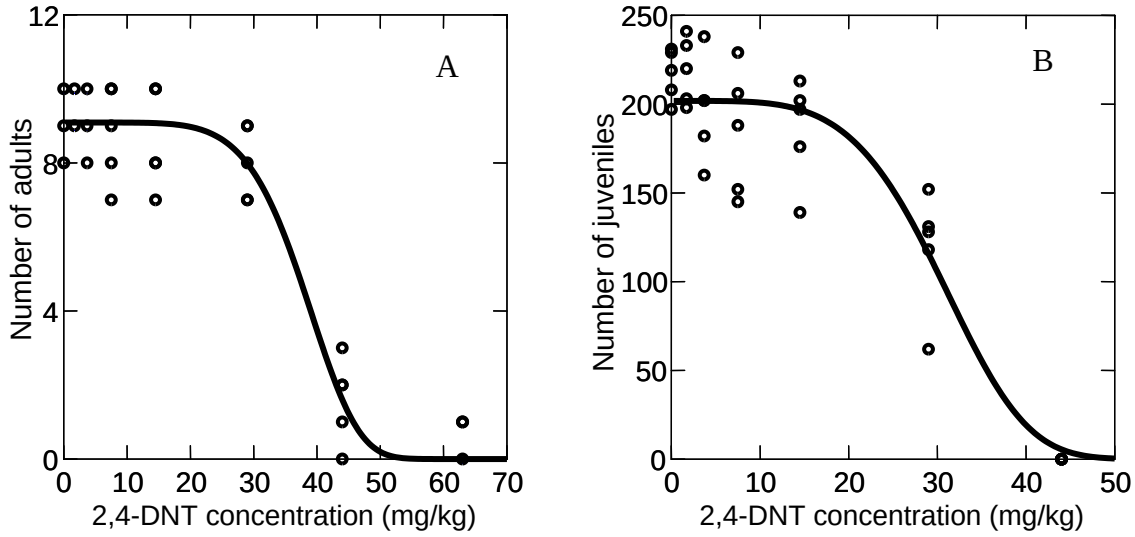


Figure 36. Effect of 2,4-DNT weathered-and-aged in Teller sandy loam soil on adult survival (A) and juvenile production (B) by *Folsomia candida.*

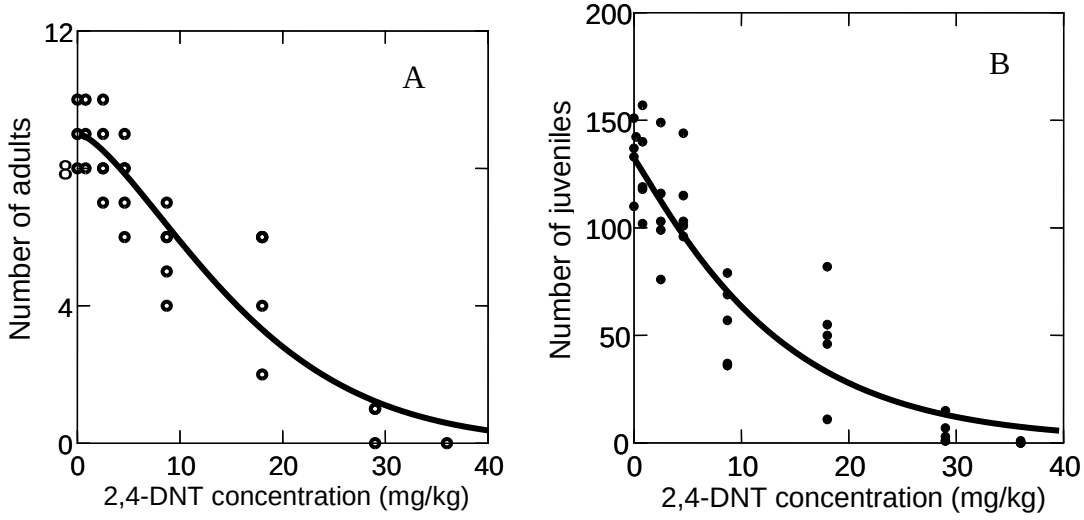


Figure 37. Effect of 2,4-DNT weathered-and-aged in Kirkland loam soil on adult survival (A) and juvenile production (B) by *Folsomia candida.*

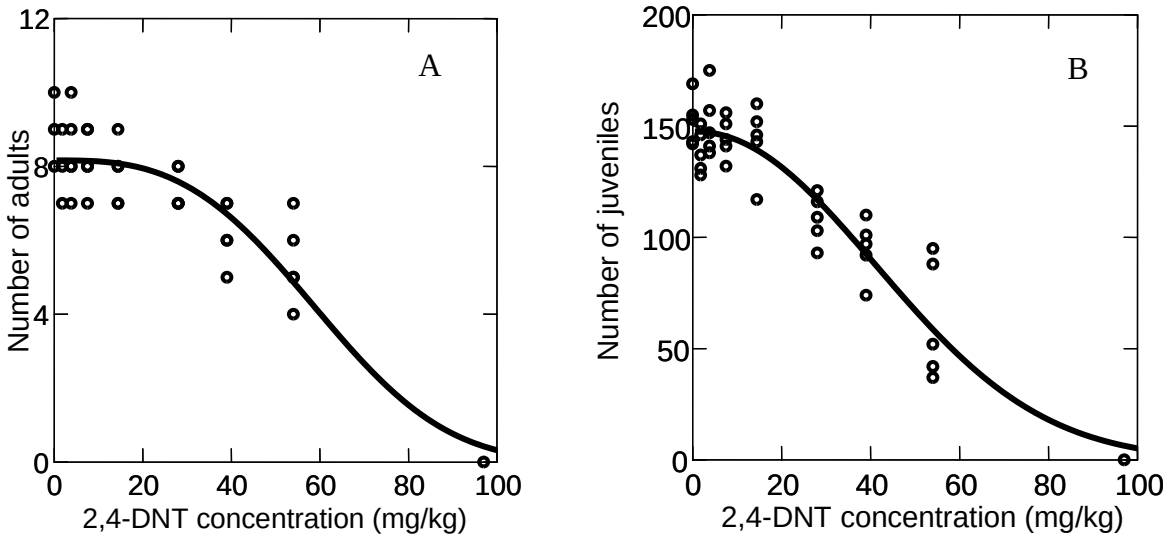


Figure 38. Effect of 2,4-DNT weathered-and-aged in Webster clay loam soil on adult survival (A) and juvenile production (B) by *Folsomia candida.*

The relationships among the 2,4-DNT toxicity benchmarks for *F. candida* and soil properties were determined using Pearson's correlation analysis. Toxicity data for *F. candida* established in our previous similarly designed studies with Sassafras sandy loam (SSL2000) soil (SERDP CU-1221) were included in this analysis. All linear correlations were performed on the original (untransformed) data. Pearson's linear correlation coefficients (r) and their respective probability (p) values are summarized in Table 43.

Table 43. Pearson correlation coefficients (r) for key soil properties and 2,4-DNT toxicity benchmarks for adult survival and reproduction endpoints determined in the definitive tests with *Folsomia candida*.

Toxicity benchmark	Clay %	p	OM %	p	pH	p	CEC cmol/kg	p
Survival								
EC_{20}	0.415	0.585	0.731	0.269	-0.091	0.909	0.383	0.617
EC_{50}	0.555	0.445	0.766	0.234	0.096	0.904	0.395	0.605
Reproduction								
EC_{20}	0.252	0.748	0.577	0.423	-0.264	0.736	0.145	0.855
EC_{50}	0.578	0.422	0.822	0.178	-0.094	0.906	0.470	0.530

Table notes: Pearson correlation coefficients with corresponding probabilities (p) were determined using data from the definitive toxicity tests with Sassafras sandy loam, Teller sandy loam, Kirkland loam, and Webster clay loam soils. Estimates of effect concentration (EC) producing a 20% (EC_{20}) or 50% (EC_{50}) decrease in the measurement endpoint compared with acetone control were determined for 2,4-DNT weathered-and-aged in soil. OM = organic matter content of the soil; CEC= cation exchange capacity of the soil.

No single soil parameter investigated directly explained the variance in toxicity of 2,4-DNT to the Collembolan *F. candida* (Table 43). An undetermined soil characteristic may affect 2,4-DNT toxicity in soil to Collembolan *F. candida.* Alternatively, the specific microenvironment of ecological niches in soil occupied by Collembola (*i.e.*, air-filled soil pores) may minimize their direct contact with chemicals in the soil pore water or within the soil solid phase, compared with the soil annelids earthworm or potworm which exhibited stronger relationships among toxicity endpoints and soil constituents.

6.3. Toxicity of 2-ADNT

6.3.1. Effects of 2-ADNT weathered-and-aged in SSL soil on the earthworms

Range-finding earthworm *Eisenia fetida* reproduction toxicity test was performed with 2-ADNT weathered-and-aged for three months in SSL2007d soil to determine the range of 2-ADNT concentrations required for the definitive toxicity test (ISO, 1998a). Nominal concentrations of 2-ADNT used in this study were 0 (negative control), 0' (acetone control), 50, 100, 200, 400, and 800 mg/kg. Corresponding analytically determined concentrations of 2-ADNT at the start of earthworm exposures in SSL2007d soil were 0, 0, 25, 51, 121, 293, and 670 mg/kg (Table 10).

Exposure to 2-ADNT weathered-and-aged in SSL2007d soil significantly (p<0.05) reduced *E. fetida* adult survival and cocoon production compared with respective values for the acetone

carrier control. The bounded NOEC and LOEC values for adult survival were 121 and 293 mg/kg, respectively (Table 44). The bounded NOEC and LOEC values for cocoon production were 25 and 51 mg/kg, respectively (Table 44). Results of this range-finding study provided sufficient data to determine concentrations of 2-ADNT for the definitive earthworm reproduction test.

Table 44. Summary of toxicological parameters for 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the range-finding toxicity test with *Eisenia fetida*.

Ecotoxicological parameters	2-ADNT (mg/kg)
Adult survival	
NOEC	121
p	0.272
LOEC	293
p	0.001
Cocoon production	
NOEC	25
p	0.100
LOEC	51
p	0.0009

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. NOEC and LOEC values were derived from Analysis of Variance and FLSD pairwise means comparison test.

Definitive 56-day reproduction toxicity tests were performed to determine the toxicity of 2-ADNT weathered-and-aged in SSL soil to the earthworm, *E. fetida*. Nominal/analytically determined (mean) treatment concentrations of 2-ADNT used in the toxicity studies were 20/10, 40/21, 60/34, 80/45, 100/58, 140/90, 200/129, 300/241, and 400/314 mg/kg (Table 11). All soil treatments were brought to 95% of the WHC 24 h prior to commencement of earthworm toxicity tests. Validity criteria were met for data produced in the Earthworm Reproduction tests. The validity criteria for the negative controls for this test are as follows: Coefficient of Variation (CV) ≤50% and adult survival ≥90% in negative controls. Adult survival in the negative controls was 100%. All NOEC, LOEC, and EC_p values were determined using analytically determined concentrations of 2-ADNT.

Adult survival, the number of cocoons produced, and the number of juveniles produced by the earthworms were significantly reduced ($p < 0.05$; compared with carrier control treatments) when *E. fetida* were exposed to 2-ADNT that was weathered-and-aged in soil. The NOEC and

LOEC values are shown in Table 45. For adult survival, cocoon production, and juvenile production, the bounded NOEC/LOEC values mg/kg (i.e., mg 2-ADNT/kg dry soil mass, DM) were 129/241, 45/58, and 45/58, respectively (Table 45).

Data resulting from earthworm reproduction tests were fit to nonlinear regression models. Adult survival and cocoon production data fit best into the Logistic model (Figure 39). Juvenile production data fit-best into the hormetic model (Figure 39). The EC_{20}/EC_{50} values for 2-ADNT (mg/kg) derived from the regression analyses for adult survival, the number of cocoons, and the number of juveniles, respectively, were: 161/204, 48/58, and 31/44 in SSL soil (Table 45).

Table 45. Summary of toxicological parameters for 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Eisenia fetida.*

Ecotoxicological parameters	2-ADNT (mg/kg)
Adult survival	
NOEC	129
P	0.274
LOEC	241
P	<0.0001
LC_{20}	161
Confidence intervals (95%)	125-198
LC_{50}	204
Confidence intervals (95%)	178-229
Model	Logistic
R^2	0.984
Cocoon production	
NOEC	45
P	0.509
LOEC	58
P	0.039
EC_{20}	48
Confidence intervals (95%)	40-56
EC_{50}	58
Confidence intervals (95%)	48-68
Model	Hormetic
R^2	0.925
Juvenile production	
NOEC	45
P	0.138
LOEC	58
P	0.012
EC_{20}	31
Confidence intervals (95%)	13-49
EC_{50}	44
Confidence intervals (95%)	30-57
Model	Logistic
R^2	0.788

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A; acetonitrile extraction (n=3)). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

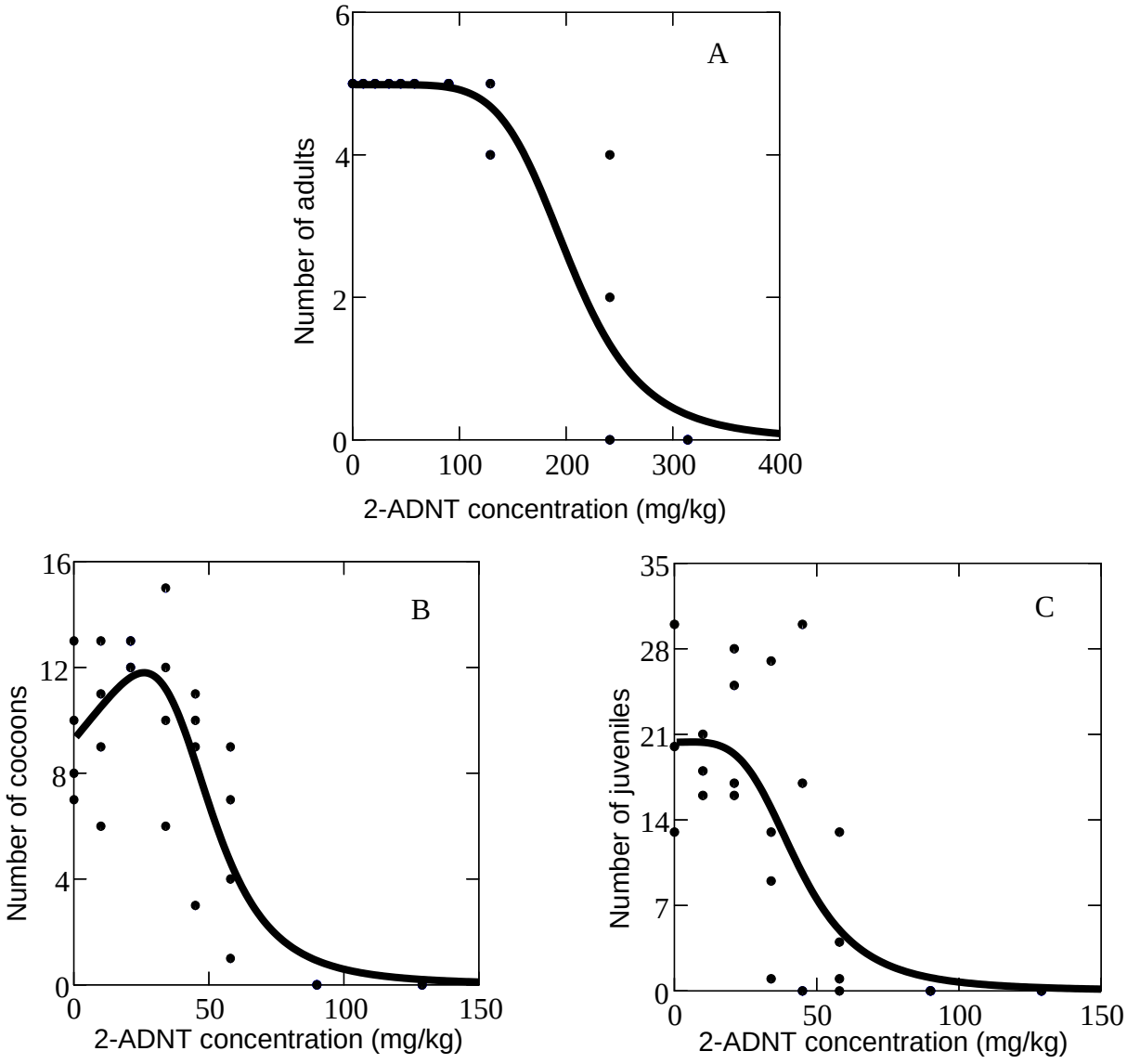


Figure 39. Effect of 2-ADNT weathered-and-aged in Sassafras sandy loam on adult survival (A), cocoon production (B), and juvenile production (C) by *Eisenia fetida.*

6.3.2. Effects of 2-ADNT weathered-and-aged in SSL soil on the potworms

A range-finding Enchytraeid Toxicity Test was performed with 2-ADNT weathered-and-aged for three months in SSL2007d soil to determine the range of 2-ADNT concentrations required for the definitive toxicity test (ISO, 2004). Nominal concentrations of 2-ADNT used in this study were 0 (negative control), 0' (acetone control), 50, 100, 200, 400, and 800 mg/kg. Corresponding analytically determined concentrations of 2-ADNT at the start of potworm exposures in SSL2007d soil were 0, 0, 25, 51, 121, 293, and 670 mg/kg (Table 10).

Results of this test complied with the validity criteria for negative control treatment, as specified in the ISO/16387 method, and those stipulated in Section 3.11.2 of this report. Survival of adult potworms was 100%, the mean number of juveniles produced was 775, and the coefficient of variation for number of juveniles was 15.5%.

The bounded NOEC and LOEC values for either adult survival or juvenile production by *E. crypticus* were 51 and 121 mg/kg, respectively (Table 46). The range of 2-ADNT concentrations selected for the test was sufficient to establish the concentration-response relationships for both the adult survival and juvenile production by *E. crypticus* (Figure 40). Nonlinear regression analyses (Gompertz model) of toxicity data yielded the respective EC_{20} and EC_{50} values (and corresponding 95% CI) mg/kg, of 57 (0-135) and 265 (101-429) for adult survival, and of 53 (22-85) and 79 (50-108) for juvenile production. Results of this range-finding study provided sufficient data to determine concentrations of 2-ADNT for the definitive Enchytraeid Toxicity Test.

The definitive Enchytraeid Toxicity Test was performed with 2-ADNT weathered-and-aged for three months in SSL2007d soil to determine the toxicity benchmark for use in deriving draft soil invertebrate-based Eco-SSL value for 2-ADNT. Nominal concentrations of 2-ADNT used in this study were 0 (negative control), 0' (acetone control), 40, 60, 100, 120, 140, 160, 180, and 400 mg/kg. Corresponding analytically determined concentrations of 2-ADNT at the start of potworm exposures in SSL2007d soil were 0, 0, 21, 34, 58, 76, 90, 99, 120, and 314 mg/kg (Table 11).

Results of this test complied with the validity criteria for negative control treatment as specified in the ISO/16387 guideline and those stipulated in Section 3.11.2 of this report. Survival of adult potworms was 100%, the mean number of juveniles produced was 941, and the coefficient of variation for number of juveniles was 11.8%. Results of definitive test with a reference toxicant, boric acid (positive control) are shown in Figure 25 and discussed in Section 4.3.1.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive test was attributable to the 2-ADNT treatments.

Table 46. Summary of toxicological parameters for 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the range-finding toxicity test with *Enchytraeus crypticus.*

Ecotoxicological parameters	2-ADNT (mg/kg)
Adult survival	
NOEC	51
p	0.838
LOEC	121
p	0.020
EC_{20}	57
Confidence intervals (95%)	0-135
EC_{50}	265
Confidence intervals (95%)	101-429
Model used	Gompertz
R^2	0.957
Juvenile production	
NOEC	51
p	0.197
LOEC	121
p	<0.0001
EC_{20}	53
Confidence intervals (95%)	22-85
EC_{50}	79
Confidence intervals (95%)	50-108
Model used	Gompertz
R^2	0.921

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. NOEC and LOEC values were derived from Analysis of Variance and FLSD pairwise means comparison test.

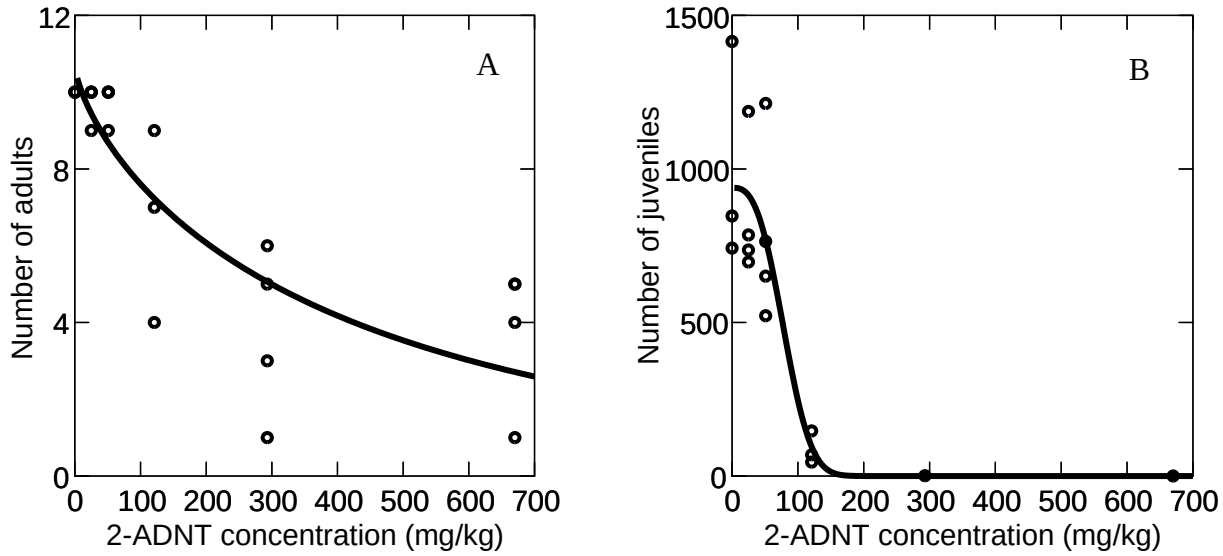


Figure 40. Effect of 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on adult survival (A) and the production of juveniles (B) by *Enchytraeus crypticus* determined in the range-finding test.

Both survival and juvenile production by adult *E. crypticus* were affected by 2-ADNT weathered-and-aged in SSL within the range of concentrations tested (Table 47). For adult survival, the bounded NOEC and LOEC values were 120 and 314 mg/kg, respectively. The EC_{20} and EC_{50} values for adult survival determined by logistic (Gompertz) model were 332 and 878 mg/kg, respectively (Table 47). Juvenile production was the more sensitive measurement endpoint for assessing 2-ADNT toxicity to *E. crypticus* than adult survival. The bounded NOEC and LOEC values for juvenile production were 76 and 90 mg/kg, respectively (Table 47). Concentration-response relationship for juvenile production determined by logistic (Gompertz) model is shown in Figure 41. The EC_{20} and EC_{50} values (mg/kg) and corresponding 95% CI for juvenile production were 76 (63-88) and 103 (95-110), respectively (Table 47).

Table 47. Summary of toxicological parameters for 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Enchytraeus crypticus.*

Ecotoxicological parameters	2-ADNT (mg/kg)
Adult survival	
NOEC	120
p	0.166
LOEC	314
p	0.003
EC_{20}	332
Confidence intervals (95%)	199-465
EC_{50}	878
Confidence intervals (95%)	43-1713
Model used	Gompertz
R^2	0.995
Juvenile production	
NOEC	76
p	0.686
LOEC	90
p	0.001
EC_{20}	76
Confidence intervals (95%)	63-88
EC_{50}	103
Confidence intervals (95%)	95-110
Model used	Gompertz
R^2	0.972

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. NOEC and LOEC values were derived from Analysis of Variance and FLSD pairwise means comparison test.

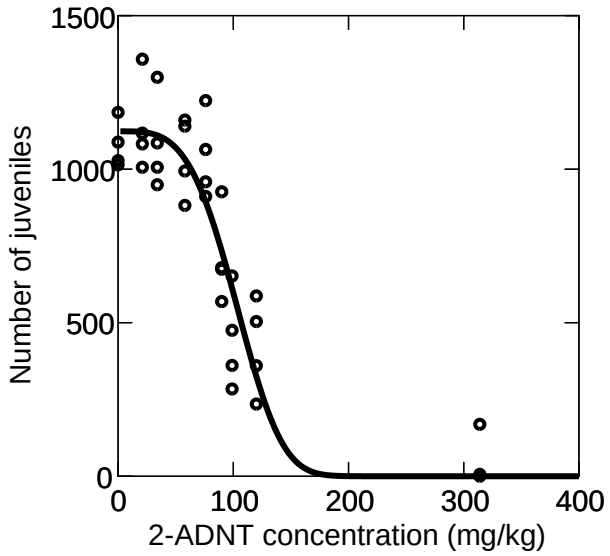


Figure 41. Effect of 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on the production of juveniles by *Enchytraeus crypticus* determined in the definitive test.

6.3.3. Effects of 2-ADNT weathered-and-aged in SSL soil on Collembola

The Folsomia Toxicity Test was used to assess the effects of 2-ADNT weathered-and-aged in SSL2007d soil on the production of juveniles and adult survival of *F. candida*. Analytically-determined 2-ADNT concentrations in soil ranged from 10 to 241 mg/kg (Table 11). Test results complied with the validity criteria for the carrier controls, as defined in the ISO/11267 test guideline and those stipulated in Section 3.11.3 of this report. Survival of adult *F. candida* was 90%, the mean number of juveniles produced was 109, and the coefficient of variation for number of juveniles was 18%. Results of definitive tests with a reference toxicant, boric acid (positive control) are shown in Figure 26 and discussed in Section 4.3.1.3. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the 2-ADNT treatments.

Ecotoxicological responses of *F. candida* to 2-ADNT weathered-and-aged in soil are shown in Table 48. Both adult survival and juvenile production were affected in 2-ADNT-amended soils within the concentration range selected for the definitive test (Figure 42). Exposure to the 2-ADNT soil treatments decreased production of juveniles and adult survival compared with respective values for the acetone carrier control (Table 48). The bounded NOEC and LOEC values for either adult survival or production of juveniles were 21 and 34 mg/kg, respectively. Logistic Gompertz model had the best fit for the adult survival data (Figure 42A). Logistic hormetic model had the best fit for the reproduction data due to stimulation of juvenile production at the lowest concentration of 2-ADNT (Figure 42B). Regression analyses of toxicity data yielded the respective EC_{20} and EC_{50} values (and corresponding 95% CI), mg/kg, of 30 (26-34) and 42 (39-46) for juvenile production; and 37 (33-41) and 55 (52-58) for adult survival.

Table 48. Summary of toxicological parameters for 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Folsomia candida*.

Ecotoxicological parameters	2-ADNT (mg/kg)
Adult survival	
NOEC	21
p	0.671
LOEC	34
p	0.039
EC_{20}	37
Confidence intervals (95%)	33-41
EC_{50}	55
Confidence intervals (95%)	52-58
Model used	Gompertz
R^2	0.991
Production of juveniles	
NOEC	21
p	0.922
LOEC	34
p	<0.0001
EC_{20}	30
Confidence intervals (95%)	26-34
EC_{50}	42
Confidence intervals (95%)	39-46
Model used	Hormetic
R^2	0.977

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

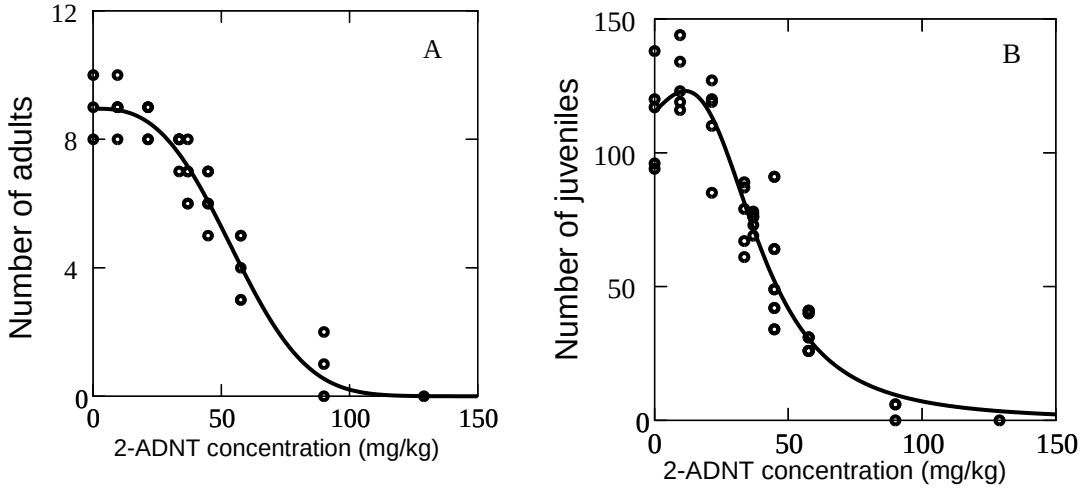


Figure 42. Effect of 2-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on adult survival (A) and the production of juveniles (B) by *Folsomia candida*.

6.4. Toxicity of 4-ADNT

6.4.1. Effects of 4-ADNT weathered-and-aged in SSL soil on the earthworms

Definitive 56-day reproduction toxicity tests were performed to determine the toxicity of 4-ADNT weathered-and-aged in SSL soil to the earthworm, *E. fetida*. Nominal/analytically determined (mean) treatment concentrations of 4-ADNT used in the toxicity studies were 20/3, 40/8, 60/13, 80/22, 100/28, 140/59, 200/75, 300/150, and 400/243 mg/kg (Table 14). All soil treatments were brought to 95% of the WHC 24 h prior to commencement of earthworm toxicity tests. Validity criteria were met for data produced in the Earthworm Reproduction tests. The validity criteria for the negative controls for this test are as follows: Coefficient of Variation (CV) ≤50% and adult survival ≥90% in negative controls. Adult survival in the negative controls was 100%. All NOEC, LOEC, and EC_p values were determined using analytically determined concentrations of 4-ADNT.

Adult survival, the number of cocoons produced, and the number of juveniles produced by the earthworms were significantly reduced ($p < 0.05$; compared with carrier control treatments) when *E. fetida* were exposed to 4-ADNT that was weathered-and-aged in soil. The NOEC and LOEC values are shown in Table 49. For adult survival, cocoon production, and juvenile production, the bounded NOEC/LOEC values mg/kg (i.e., mg 4-ADNT/kg dry soil mass, DM) were 59/75, 13/22, and 8/13, respectively (Table 49).

Data resulting from earthworm reproduction tests were fit to nonlinear regression models. Adult survival, cocoon production, and juvenile production data fit best into the Logistic model (Figure 43). The EC_{20}/EC_{50} values for 4-ADNT (mg/kg) derived from the regression analyses for adult survival, the number of cocoons and the number of juveniles, respectively were: 52/69, 17/27, and 12/20 (Table 49).

Table 49. Summary of toxicological parameters for 4-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Eisenia fetida.*

Ecotoxicological parameters	4-ADNT (mg/kg)
Adult survival	
NOEC	59
p	1.0000
LOEC	75
p	<0.0001
LC_{20}	52
Confidence intervals (95%)	27-77
LC_{50}	69
Confidence intervals (95%)	59-79
Model	Logistic
R^2	0.987
Cocoon production	
NOEC	13
p	0.143
LOEC	22
p	0.002
EC_{20}	17
Confidence intervals (95%)	12-22
EC_{50}	27
Confidence intervals (95%)	22-32
Model	Logistic
R^2	0.956
Juvenile production	
NOEC	8
p	0.338
LOEC	13
p	0.025
EC_{20}	12
Confidence intervals (95%)	7-18
EC_{50}	20
Confidence intervals (95%)	15-26
Model	Logistic
R^2	0.928

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A; acetonitrile extraction (n=3)). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

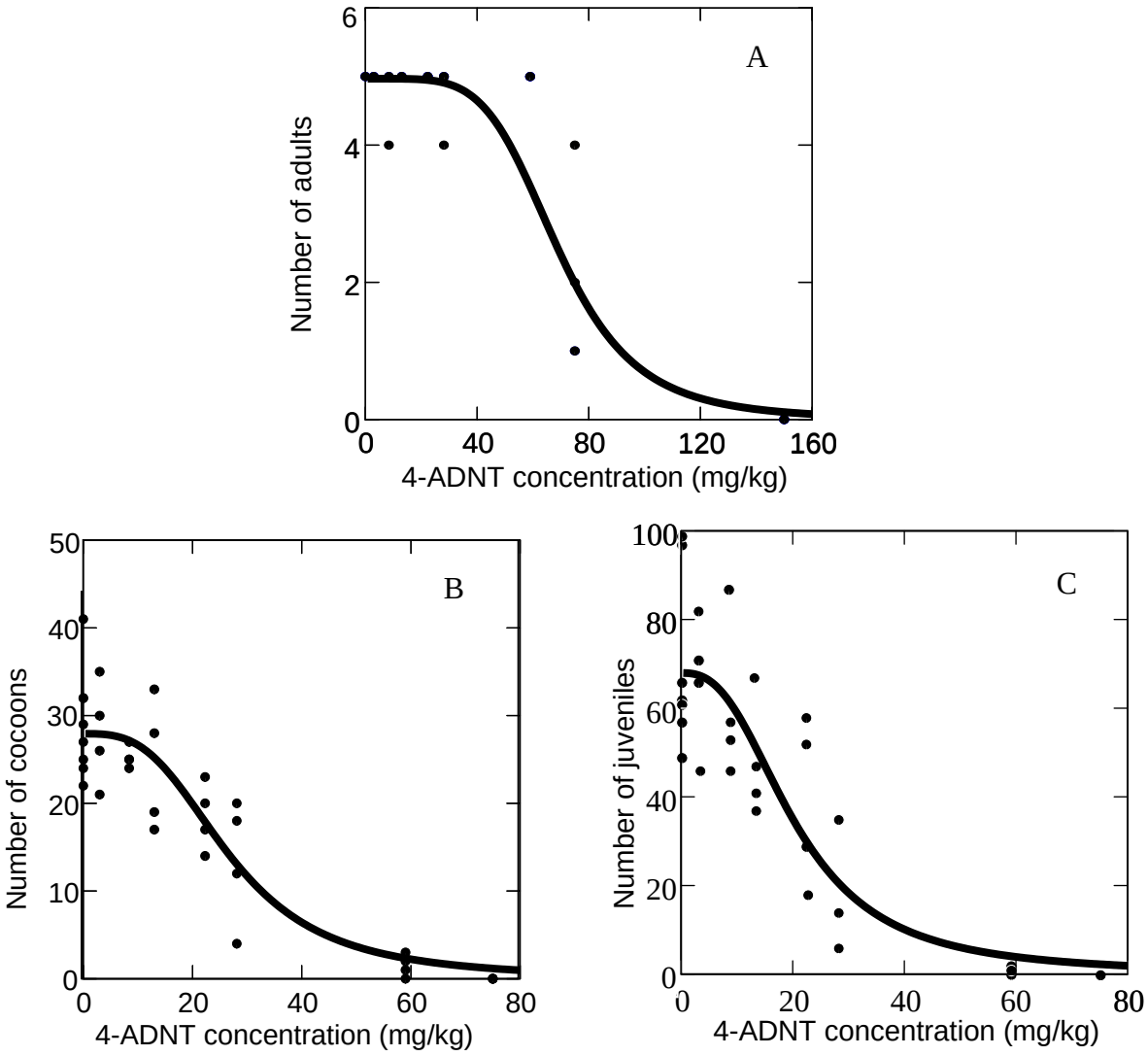


Figure 43. Effect of 4-ADNT weathered-and-aged in Sassafras sandy loam on adult survival (A), cocoon production (B), and juvenile production (C) by *Eisenia fetida.*

6.4.2. Effects of 4-ADNT weathered-and-aged in SSL soil on the potworms

The definitive Enchytraeid Toxicity Test was performed with 4-ADNT weathered-and-aged in SSL2007d soil to determine the toxicity benchmark for use in deriving draft soil invertebrate-based Eco-SSL value for 4-ADNT. Nominal concentrations of 4-ADNT used in this study were 0 (negative control), 0' (acetone control), 60, 100, 160, 200, 300, and 400 mg/kg. Corresponding analytically determined concentrations of 4-ADNT at the start of potworm exposures in SSL2007d soil were 0, 0, 13, 28, 63, 75, 150, and 243 mg/kg (Table 14). Results of this test complied with the validity criteria for negative control treatment as specified in the ISO/16387 test guideline and those stipulated in Section 3.11.2 of this report. Survival of adult potworms

was 98%, the mean number of juveniles produced was 304, and the coefficient of variation for number of juveniles was 17.3%. Results of definitive test with a reference toxicant, boric acid (positive control) are shown in Figure 25 and discussed in Section 4.3.1.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive test was attributable to the 4-ADNT treatments.

Both survival and juvenile production by adult *E. crypticus* were affected by 4-ADNT weathered-and-aged in SSL within the range of concentrations tested (Table 50). The range of 4-ADNT concentrations selected for the test was sufficient to establish the concentration-response relationships for both the adult survival and juvenile production by *E. crypticus* (Figure 44). For adult survival, the bounded NOEC and LOEC values were 75 and 150 mg/kg, respectively. The EC_{20} and EC_{50} values for adult survival determined by logistic (Gompertz) model were 137 and 185 mg/kg, respectively (Table 50). Juvenile production was the more sensitive measurement endpoint for assessing 4-ADNT toxicity to *E. crypticus* than adult survival. The bounded NOEC and LOEC values for juvenile production were 13 and 63 mg/kg, respectively (Table 50). The EC_{20} and EC_{50} values (and corresponding 95% CI), mg/kg, for juvenile production were 21 (9-32) and 37 (26-47), respectively (Table 50).

Table 50. Summary of toxicological parameters for 4-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Enchytraeus crypticus.*

Ecotoxicological parameters	4-ADNT (mg/kg)
Adult survival	
NOEC	75
p	0.749
LOEC	150
p	0.002
EC_{20}	137
Confidence intervals (95%)	115-159
EC_{50}	185
Confidence intervals (95%)	169-202
Model used	Gompertz
R^2	0.988
Juvenile production	
NOEC	13
p	0.127
LOEC	63
p	<0.0001
EC_{20}	21
Confidence intervals (95%)	9-32
EC_{50}	37
Confidence intervals (95%)	26-47
Model used	Gompertz
R^2	0.979

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. NOEC and LOEC values were derived from Analysis of Variance and FLSD pairwise means comparison test.

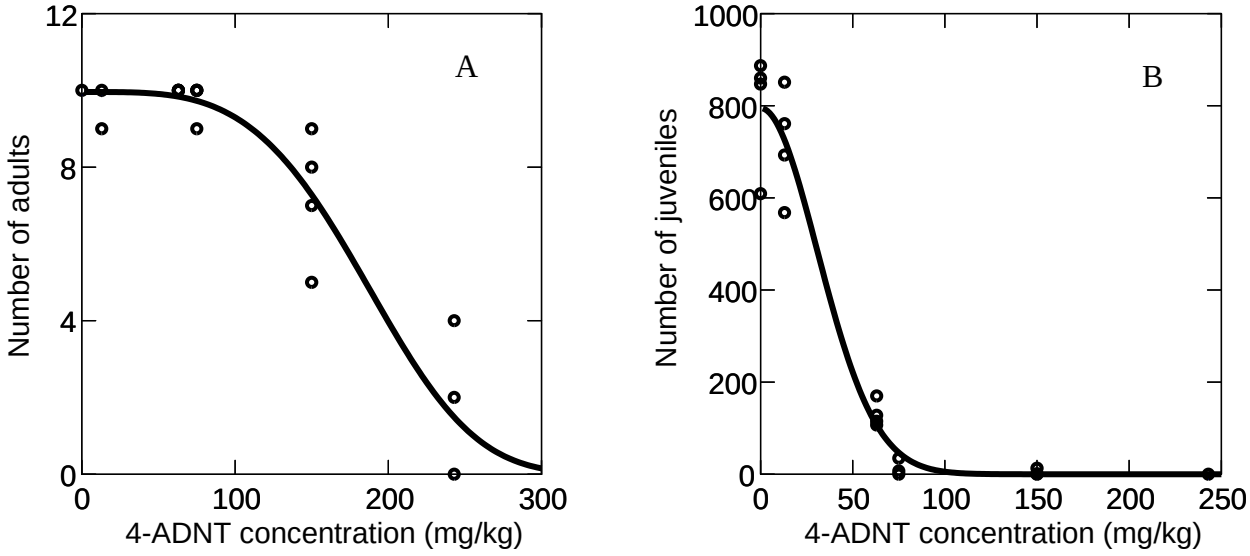


Figure 44. Effect of 4-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on adult survival (A) and the production of juveniles (B) by *Enchytraeus crypticus*.

6.4.3. Effects of 4-ADNT weathered-and-aged in SSL soil on Collembola

The Folsomia Toxicity Test was used to assess the effects of 4-ADNT weathered-and-aged in SSL2007d soil on the adult survival and production of juveniles of *F. candida*. Analytically-determined 4-ADNT concentrations in soil ranged from 3 to 150 mg/kg (Table 14). Test results complied with the validity criteria for the negative controls, as defined in the ISO/11267 test guideline and those stipulated in Section 3.11.3 of this report. Survival of adult *F. candida* was 94%, the mean number of juveniles produced was 121, and the coefficient of variation for number of juveniles was 13.9%. Results of definitive tests with a reference toxicant, boric acid (positive control), are shown in Figure 26 and discussed in Section 4.3.1.3. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the 4-ADNT treatments.

Ecotoxicological responses of *F. candida* to 4-ADNT weathered-and-aged in each soil are shown in Table 51. Both adult survival and juvenile production were affected in 4-ADNT-amended soils within the concentration ranges selected for definitive tests. Exposure to the 4-ADNT soil treatments decreased adult survival and production of juveniles compared with respective values for the acetone carrier control. The bounded NOEC and LOEC values for either adult survival or production of juveniles were 22 and 28 mg/kg, respectively (Table 51). Logistic Gompertz model had the best fit for either adult survival (Figure 45A) or juvenile production (Figure 45B) data from toxicity tests with 4-ADNT weathered-and-aged in SSL2007d soil. Regression analyses of toxicity data yielded the respective EC_{20} and EC_{50} values (and corresponding 95%

CI), mg/kg, of 22 (11-32) and 55 (42-67) for adult survival, and of 26 (19-33) and 47 (41-53) for juvenile production (Table 51).

Table 51. Summary of toxicological parameters for 4-ADNT weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Folsomia candida*.

Ecotoxicological parameters	4-ADNT (mg/kg)
Adult survival	
NOEC	22
p	1.0
LOEC	28
p	0.004
EC_{20}	22
Confidence intervals (95%)	11-32
EC_{50}	55
Confidence intervals (95%)	42-67
Model used	Gompertz
R^2	0.970
Production of juveniles	
NOEC	22
p	0.785
LOEC	28
p	<0.0001
EC_{20}	26
Confidence intervals (95%)	19-33
EC_{50}	47
Confidence intervals (95%)	41-53
Model used	Gompertz
R^2	0.981

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

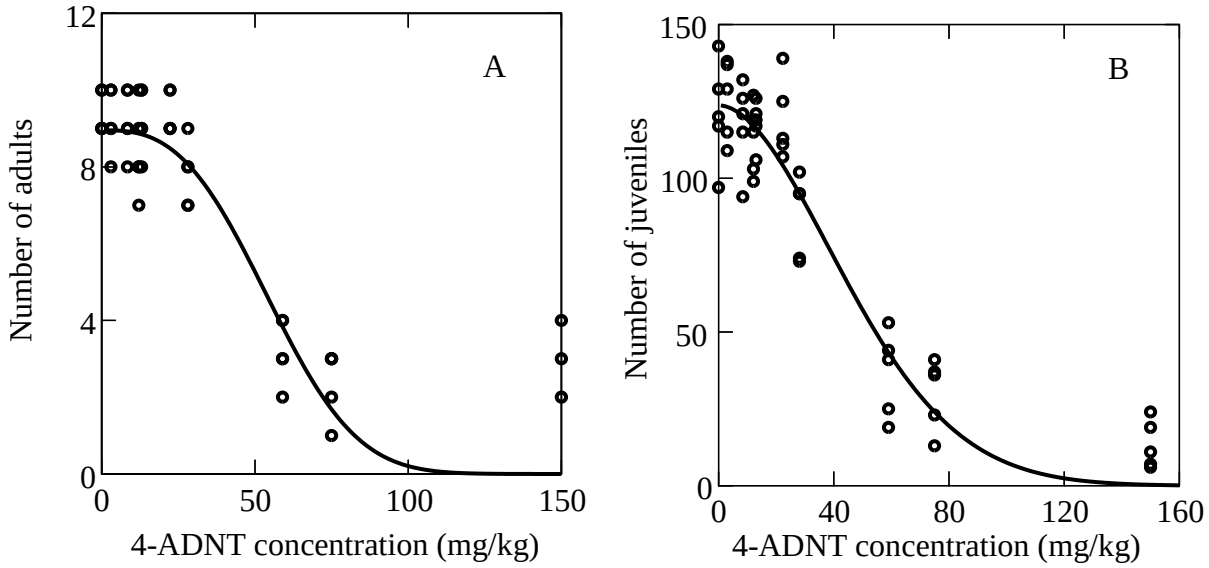


Figure 45. Effect of 4-ADNT weathered-and-aged in Sassafras sandy loam soil on adult survival (A) and production of juveniles (B) by *Folsomia candida.*

6.5. Toxicity of HMX

6.5.1. Effects of HMX weathered-and-aged in TSL soil on the earthworms

The composite range-finding/limit reproduction test described above was conducted to determine the effects of HMX weathered-and-aged in TSL on the earthworm *E. fetida*. Nominal concentrations of HMX in soil for this test were 0 (negative control), 0' (acetone control), 100, 1000, 5000, and 10000 mg/kg. The respective analytically determined HMX concentrations were 0, 0', 72, 913, 4888, and 10208 mg/kg (Table 17). Results showed that adult survival in all HMX treatments was not significantly different ($p>0.05$) compared with acetone carrier control. The earthworm survival rates were 100, 100, 95, 100, and 97.5 percent in the respective carrier control and soil treatments, with analytically determined HMX concentrations, of 72, 913, 4888, and 10208 mg/kg. Results of the statistical analyses of the reproduction portion of the test were inconclusive. The production of cocoons was significantly ($p<0.05$) decreased at 72, 913, and 10208 mg/kg, but not at 4888 mg/kg, compared with carrier control. The production of juveniles was significantly ($p<0.05$) decreased only at 913 mg/kg. Data for either cocoon or juvenile production did not fit any of the conventional nonlinear regression models (exponential, Gompertz, or hormetic). The data were fit to a linear model, which estimated the respective EC_{20} (95% CI) and EC_{50} (95% CI) concentrations, mg/kg, of 4250 (1848-6651) and 10624 (4620-16628) for cocoon production; and 7746 (0-22117) and 19367 (0-55293) for juvenile production. These estimates imparted low confidence in the test results due to a wide range of the 95% CI, and because estimated EC_{50} concentrations were extrapolated outside the range of HMX treatment concentrations of the test. Therefore, this test was repeated to determine the toxicological values for cocoon and juvenile production. The values determined in the range-finding/Limit Test for adult survival were valid and used for the NOEC/LOEC determinations.

Nominal HMX concentrations selected for the repeat range-finding/Limit Test included 0 (negative control), 0' (acetone control), 5, 10, 20, 40, 60, 80, 100, 200, and 400 mg/kg. The respective analytically determined HMX concentrations in TSL at the end of weathering-and-aging were 0, 0, 5, 9, 18, 39, 57, 79, 98, 187, and 499 mg/kg (Table 18). All soil treatments were brought to 95% of the WHC 24 h prior to commencement of earthworm toxicity tests. Validity criteria were met for data produced in the Earthworm Reproduction tests. The validity criteria for the negative controls for this test are as follows: Coefficient of Variation (CV) ≤50% and adult survival ≥90% in negative controls. Adult survival in the negative controls was 100%. All NOEC, LOEC, and EC_p values were determined using analytically determined concentrations of HMX.

The production of cocoons and the production of juveniles by the earthworms were significantly reduced ($p<0.05$; compared with carrier control treatments) when *E. fetida* were exposed to HMX that was weathered-and-aged in TSL soil. The NOEC and LOEC values are shown in Table 52. Cocoon production was significantly decreased, compared with carrier control, in the first positive HMX concentration tested, producing an unbounded LOEC of 5 mg/kg, For juvenile production, the bounded NOEC and LOEC values (mg/kg dry soil mass) for HMX were 5 and 9, respectively (Table 52). Adult survival was not significantly ($p>0.05$) affected by HMX at the highest concentration tested in this study, producing an unbounded NOEC value for adult survival of 10208 mg/kg.

Data resulting from earthworm reproduction tests were fit to nonlinear regression models. Cocoon production and juvenile production data fit best into the Exponential model (Figure 46). The EC_{20}/EC_{50} values for HMX (mg/kg) derived from the regression analyses for the production of cocoons and the production of juveniles, respectively, were: 2/6, and 2/8 (Table 52).

Table 52. Summary of toxicological parameters for HMX weathered-and-aged in Teller sandy loam (TSL2002) soil determined in toxicity test with *Eisenia fetida*.

Ecotoxicological parameters	HMX (mg/kg)
Adult survival	
NOEC	10208[†]
p	0.146
LOEC	>10208
p	0.146
LC_{20}	ND
Confidence intervals (95%)	ND
LC_{50}	ND
Confidence intervals (95%)	ND
Model	ND
R^2	ND
Cocoon production	
NOEC	<5
p	1.000
LOEC	5[††]
p	0.003
EC_{20}	2
Confidence intervals (95%)	0.6-3.3
EC_{50}	6
Confidence intervals (95%)	1.8-10.3
Model	Exponential
R^2	0.883
Juvenile production	
NOEC	5
p	0.340
LOEC	9
p	0.047
EC_{20}	2
Confidence intervals (95%)	0.5-4.4
EC_{50}	8
Confidence intervals (95%)	1.5-13.7
Model	Exponential
R^2	0.742

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A; acetonitrile extraction (n=3)). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. [†]=unbounded NOEC; [††]=unbounded LOEC; ND = Not Determined; Adult survival was 100%, therefore; EC values for adult survival could not be determined within the concentration range tested.

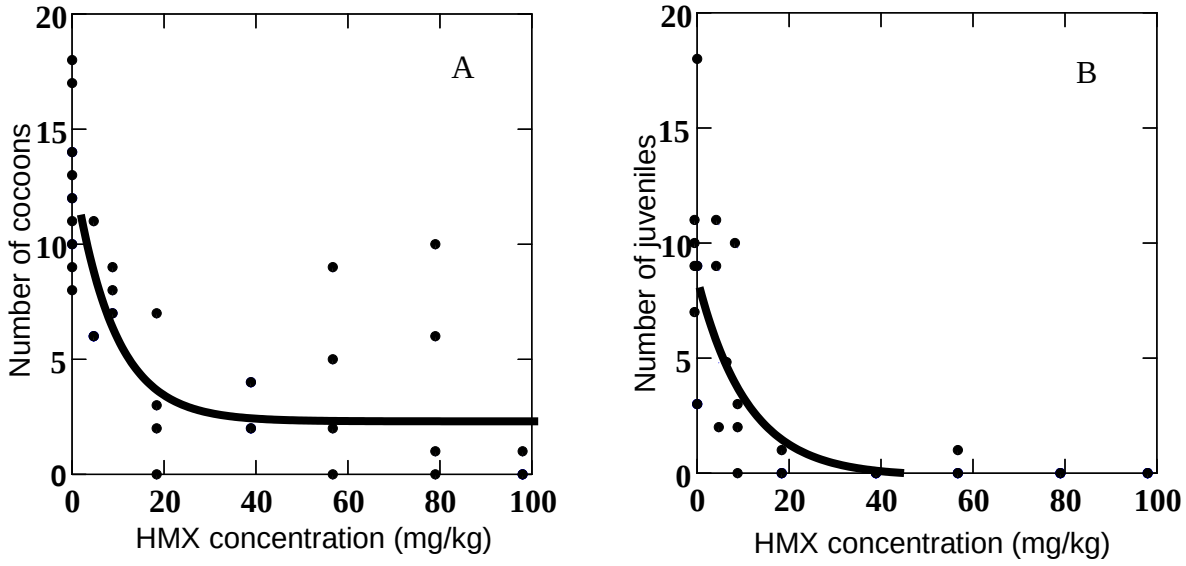


Figure 46. Effect of HMX weathered-and-aged in Teller sandy loam on cocoon production (A) and juvenile production (B) by *Eisenia fetida.*

6.5.2. Effects of HMX weathered-and-aged in TSL soil on the potworms

The potworm *E. crypticus* composite toxicity/Limit Test with HMX weathered-and-aged in TSL soil for three months was conducted using nominal HMX concentrations of 0 (negative control), 0' (acetone control), 100, 1000, 5000, and 10000 mg/kg. The respective analytically determined HMX concentrations at the start of the test were 0, 0', 72, 913, 4888, and 10208 mg/kg (Table 17). The following replication was used: eight replicates in 0' (acetone carrier control) and 10208 mg/kg treatments, and four replicates in the 0 (negative control), 72, 913, and 4888 mg/kg treatments. The results of the present study complied with the validity criteria for negative control treatment specified in the ISO/16387 guideline and those stipulated in Section 3.11.2 of this report. Survival of *E. crypticus* adults was 93%, the mean number of juveniles produced was 412, and the coefficient of variation for number of juveniles was 27%. Results of definitive test with a reference toxicant, boric acid (positive control) are shown in Figure 25 and discussed in Section 4.3.1.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive test was attributable to the HMX treatments.

Adult potworms were harvested and counted after 14 days. Results showed no statistically significant (p>0.05) effect of HMX on adult potworm survival in any of the treatments tested in TSL soil compared with the acetone carrier control. The adult potworm survival rates were 93, 98, 93, 100, 100, and 100 percent in HMX treatments 0, 0', 72, 913, 4888, and 10208 mg/kg, respectively. The reproduction portion of the test was completed after an additional 14 days of exposure. Production of juveniles by *E. crypticus* in the greatest HMX treatment of 10208 mg/kg was not significantly different (t-Test; p=0.535) from that in carrier control. These results showed that exposure to HMX weathered-and-aged in TSL for three months did not affect either

adult potworm survival or the production of juveniles at≤10208 mg/kg (unbounded NOEC), thus confirming the toxicity data previously established in our studies with SSL soil (SERDP CU-1221).

6.5.3. Effects of HMX weathered-and-aged in TSL soil on Collembola

The Collembolan *F. candida* composite toxicity/Limit Test with HMX weathered-and-aged in TSL soil for three months was conducted using nominal HMX concentrations of 0 (negative control), 0' (acetone carrier control), 100, 1000, 5000, and 10000 mg/kg. The respective analytically determined HMX concentrations at the start of the tests were 0, 0', 72, 913, 4888, and 10208 mg/kg (Table 17). The experimental design included five replicates for negative control, 72, 913, and 4888 mg/kg treatments, and 10 replicates of the carrier control and 10208 mg/kg treatments. The results of the present study complied with the validity criteria for the negative control treatment specified in the ISO/11267 method. Survival of *F. candida* adults was 89%, the mean number of juveniles produced was 100, and the coefficient of variation for number of juveniles was 15%.

Adults and juveniles, respectively, were counted after 28 days of exposure. Results showed that adult survival in all positive HMX treatments of TSL soil was not significantly different (p>0.05) compared to the carrier control, with respective survival rates of 90, 88, 86, 88, and 88 percent for HMX treatment concentrations (mg/kg) 0', 72, 913, 4888, and 10208. Juvenile production by *F. candida* was not significantly (p>0.05) affected by exposure to HMX weathered-and-aged in TSL soil compared with the carrier control. The average numbers of juveniles were 106, 110, 101, 110, and 117 in HMX treatments 0', 72, 913, 4888, and 10208 mg/kg, respectively. These results showed that exposure to HMX weathered-and-aged in TSL for three months did not affect either the survival of adult *F. candida* or the production of juveniles at ≤10208 mg/kg (unbounded NOEC).

6.6. Toxicity of NG

6.6.1. Effects of NG on the earthworms in SSL soil

A range-finding Earthworm Reproduction Test was conducted with NG freshly amended into Sassafras sandy loam (SSL2004) soil. Procedures for this test were the same as those described for the definitive tests with 2,4-DNT *except*: three replicates were prepared per treatment concentration, the test was terminated after 28 d, and the number of cocoons produced was used as the single reproduction measurement endpoint. Nominal concentrations selected for tests with NG were 0 (acetone control), 1, 10, 100, 1000, and 5000 mg/kg dry SSL soil. Corresponding analytically determined concentrations of NG in SSL soil are reported in Table 19.

Adult survival was not significantly reduced at ≤85 mg NG/kg soil. At the end of this study there were no survivors in soil containing either 898 or 4558 mg NG/kg soil. The number of cocoons produced by *E. fetida* in SSL soil was not significantly (p>0.05) reduced in soil with NG concentrations at ≤5 mg/kg compared with acetone carrier control. Cocoon production was significantly (p<0.05) decreased (77% reduction from the number in control) in soil with NG concentration of 85 mg/kg. No cocoons were produced at concentrations of 898 and 4558 mg/kg.

Results from this study were used to determine soil concentrations for each of the treatment levels in the definitive Earthworm Reproduction Test.

Definitive 56-day reproduction toxicity test was performed to determine the toxicity of NG weathered-and-aged in SSL (SSL2007d) soil to the earthworm, *E. fetida*. Nominal/analytically determined (mean) positive treatment concentrations (mg/kg) of NG used in the toxicity studies were 5/BDL, 50/0.6, 75/2.1, 100/1.8, 160/6.2, 200/21, 250/22, 300/36, 400/122 and 650/268, respectively (Table 21). All soil treatments were brought to 95% of the SSL soil WHC 24 h prior to commencement of earthworm toxicity tests. Validity criteria were met for data produced in the Earthworm Reproduction tests. The validity criteria for the negative controls for this test are as follows: Coefficient of Variation (CV) ≤50% and adult survival ≥90% in negative controls. Adult survival in the negative controls was 100%. All NOEC, LOEC, and EC_p values were determined using analytically determined concentrations of NG.

Adult survival, the number of cocoons produced, and the number of juveniles produced by the earthworms were significantly reduced ($p < 0.05$; compared with carrier control treatments) when *E. fetida* were exposed to NG that was weathered-and-aged in SSL soil. The NOEC and LOEC values are shown in Table 53. For adult survival, cocoon production, and juvenile production, the bounded NOEC/LOEC values, mg/kg (i.e., mg NG/kg dry soil mass, DM), were 122/268, 36/122, and 36/122, respectively (Table 53).

Data resulting from earthworm reproduction tests were fit to regression models. Adult survival and cocoon production data fit best into the Linear model, and juvenile production data fit best into the Logistic model (Figure 47). The EC_{20}/EC_{50} values for NG (mg/kg) derived from the regression analyses for adult survival, the number of cocoons and the number of juveniles, respectively were: 63/157, 24/60, and 21/26 (Table 53).

Table 53. Summary of toxicological parameters for NG weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Eisenia fetida.*

Ecotoxicological parameter	NG (mg/kg)
Adult survival	
NOEC	122
p	0.060
LOEC	268
p	0.0001
LC_{20}	63
Confidence intervals (95%)	55-71
LC_{50}	157
Confidence intervals (95%)	137-177
Model	Linear
R^2	0.984
Cocoon production	
NOEC	36
p	0.408
LOEC	122
p	0.0004
EC_{20}	24
Confidence intervals (95%)	14-34
EC_{50}	60
Confidence intervals (95%)	36-85
Model	Linear
R^2	0.828
Juvenile production	
NOEC	36
p	0.066
LOEC	122
p	0.004
EC_{20}	21
Confidence intervals (95%)	11-31
EC_{50}	26
Confidence intervals (95%)	13-39
Model	Hormetic
R^2	0.733

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A; acetonitrile extraction (n=3)). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

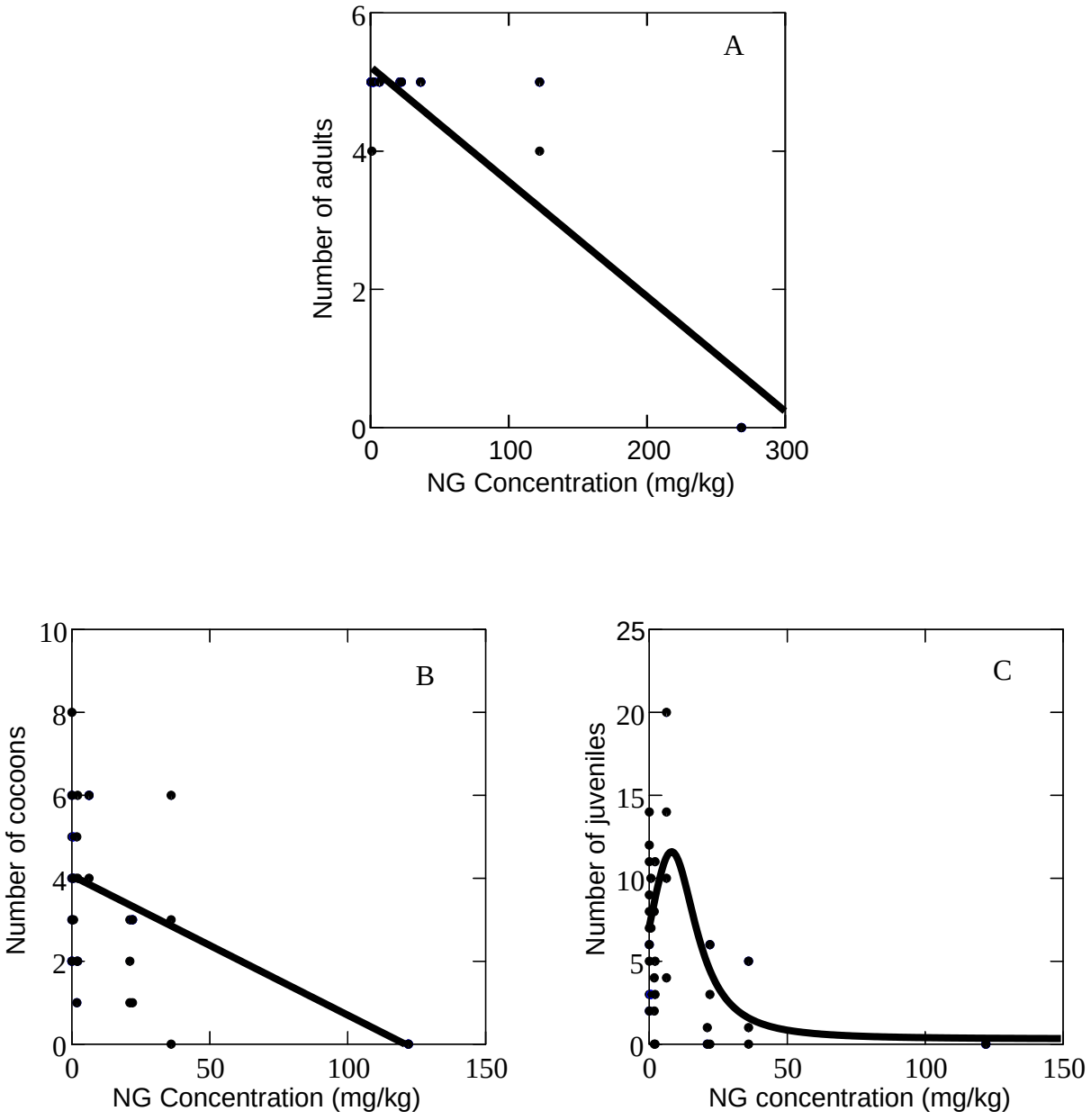


Figure 47. Effect of NG weathered-and-aged in Sassafras sandy loam on adult survival (A), cocoon production (B), and juvenile production (C) by *Eisenia fetida.*

6.6.2. Effects of NG on the potworms in SSL soil

A range-finding Enchytraeid Toxicity Test was conducted with NG freshly amended into SSL2004 to determine treatment concentrations for the definitive test. Procedures for this test were the same as those described for the definitive tests with 2,4-DNT. Nominal concentrations selected for test with NG in SSL soil were 0 (acetone carrier control), 1, 10, 100, 1000, and 5000 mg/kg. Corresponding analytically determined concentrations of NG in SSL soil are reported in Table 19.

Results of the range-finding study complied with the validity criteria for control treatment specified in the ISO/16387 method. Survival of adult potworms was 100%, the mean number of juveniles produced was 1206, and the coefficient of variation for number of juveniles was 30%. Numbers of surviving adult *E. crypticus* were not significantly (p>0.05) different among carrier control, 0.84, 4.9, and 85 mg/kg treatments. No adults survived in 898 and 4558 mg/kg treatments (Table 54). Juvenile production was not significantly (p>0.05) affected by NG concentrations of 0.84, 4.9, and 85 mg/kg compared with number of juveniles in carrier control. No juveniles were produced in 898 or 4558 mg/kg treatments. These results showed that neither adult survival nor juvenile production by *E. crypticus* was affected by exposure to NG in SSL soil at ≤85 mg/kg, while 100% adverse effect for either measurement endpoint was determined at the greater NG concentrations of 898 and 4558 mg/kg that were tested in this range-finding study. An additional study was conducted using nominal NG concentrations 0 (negative control), 0’ (acetone control), 100, 200, 400, 600, and 800 mg/kg in freshly amended SSL2007d soil. Corresponding analytically determined concentrations of NG in SSL2007d soil were 0, 0, 92, 202, 404, 564, and 797 mg/kg.

Results of this test complied with the validity criteria for the control treatment as specified in the ISO/16387 method. In control treatments survival of adult potworms was 98%, the mean number of juveniles produced was 948, and the coefficient of variation for number of juveniles was 14%. Numbers of surviving adult *E. crypticus* were not significantly (p>0.05) different among the acetone carrier control, 92, and 202 mg/kg treatments. No adults survived in NG treatment concentrations ≥404 mg/kg. Juvenile production was significantly (p<0.0001) decreased in the first positive NG concentration compared with the number of juveniles in the carrier control, producing an unbounded LOEC of 92 mg/kg (Table 54). No juveniles were produced in 898 or 4558 mg/kg treatments. The range of NG concentrations selected for the repeat test was sufficient to establish the concentration-response relationship based on juvenile production by *E. crypticus* (Figure 48). Nonlinear regression analysis of toxicity data yielded the EC_{20} and EC_{50} values and corresponding 95% confidence intervals (CI) for juvenile production of 30 (13-46) and 76 (59-93) mg/kg, respectively. These results were used to determine concentrations for the definitive testing of NG weathered-and-aged in SSL2007d soil.

Table 54. Summary of toxicological parameters for NG freshly amended into Sassafras sandy loam (SSL2007d) soil determined in the range-finding toxicity test with *Enchytraeus crypticus.*

Ecotoxicological parameters	NG (mg/kg)
Adult survival	
NOEC	202
p	1.0
LOEC	404
p	<0.0001
Juvenile production	
NOEC	<92
P	ND
LOEC (unbounded)	92
p	<0.0001
EC_{20}	30
Confidence intervals (95%)	13-46
EC_{50}	76
Confidence intervals (95%)	59-93
Model used	Gompertz
R^2	0.988

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). ND=not determined; could not be determined within the concentration range tested; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. NOEC and LOEC values were derived from Analysis of Variance and FLSD pairwise means comparison test.

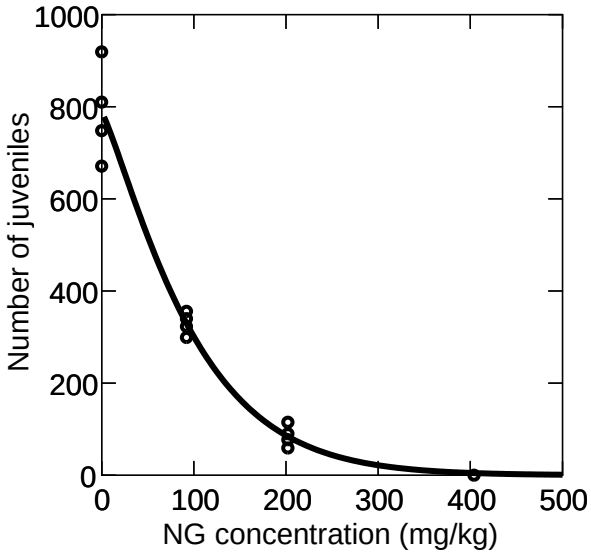


Figure 48. Effect of NG on juvenile production by *Enchytraeus crypticus* in freshly amended Sassafras sandy loam (SSL2007d) soil.

The definitive Enchytraeid Toxicity Test was performed with NG weathered-and-aged in SSL2007d soil to determine the toxicity benchmark for use in deriving draft soil invertebrate-based Eco-SSL value for NG. Nominal concentrations of NG used in this study were 0 (negative control), 0' (acetone carrier control), 100, 250, 300, and 400 mg/kg. Corresponding analytically determined concentrations of NG at the start of potworm exposures in SSL2007d soil were 0, 0, 1.8, 22, 36, and 122 mg/kg (Table 21). Results of this test complied with the validity criteria for negative control treatment as specified in the ISO/16387 test guideline and those stipulated in Section 3.11.2 of this report. Survival of adult potworms was 98%, the mean number of juveniles produced was 912, and the coefficient of variation for number of juveniles was 19%. Results of definitive test with a reference toxicant, boric acid (positive control) are shown in Figure 25 and discussed in Section 4.3.1.2. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive test was attributable to the NG treatments.

Juvenile production by adult *E. crypticus* was affected by NG weathered-and-aged in SSL2007d within the range of concentrations tested (Table 55). The range of NG concentrations selected for the test was sufficient to establish the concentration-response relationships for juvenile production by *E. crypticus* (Figure 49). Survival of *E. crypticus* adults was not affected by NG up to and including the greatest concentration tested in this study, producing an unbounded NOEC of 122 mg/kg. Juvenile production was the more sensitive measurement endpoint for assessing NG toxicity to *E. crypticus* than adult survival. The bounded NOEC and LOEC values for juvenile production were 36 and 122 mg/kg, respectively (Table 55). The EC_{20} and EC_{50} values (and corresponding 95% CI for juvenile production), mg/kg, were 44 (11-77) and 146 (82-211), respectively (Table 55).

Table 55. Summary of toxicological parameters for NG weathered-and-aged in Sassafras sandy loam (SSL2007d) soil determined in the definitive toxicity test with *Enchytraeus crypticus*.

Ecotoxicological parameters	NG (mg/kg)
Adult survival	
NOEC	122[†]
p	0.056
LOEC	>122
p	ND
EC_{20}	>122
Confidence intervals (95%)	ND
EC_{50}	>122
Confidence intervals (95%)	ND
Model used	ND
R^2	ND
Juvenile production	
NOEC	36
p	0.136
LOEC	122
p	<0.0001
EC_{20}	44
Confidence intervals (95%)	11-77
EC_{50}	146
Confidence intervals (95%)	82-211
Model used	Gompertz
R^2	0.986

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. [†]Unbounded NOEC; NOEC and LOEC values were derived from Analysis of Variance and FLSD pairwise means comparison test. ND=not determined; could not be determined within the concentration range tested.

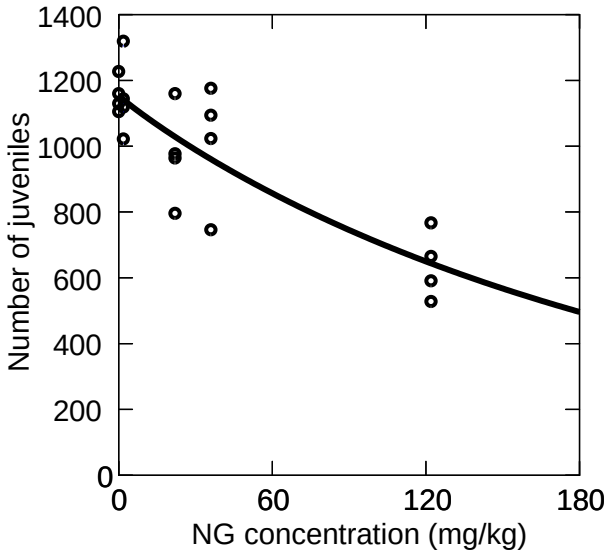


Figure 49. Effect of NG weathered-and-aged in Sassafras sandy loam (SSL2007d) soil on juvenile production by *Enchytraeus crypticus*.

6.6.3. Effects of NG weathered-and-aged in SSL soil on Collembola

The Folsomia Toxicity Test was used to assess the effects of NG weathered-and-aged in SSL2007d soil on the production of juveniles and adult survival of *F. candida*. Analytically-determined NG positive exposure concentrations for these definitive toxicity tests in SSL soil ranged from 0.2 to 36 mg/kg. Test results complied with the validity criteria for the carrier controls, as defined in the ISO/11267 test guideline, and those stipulated in Section 3.11.3 of this report. Survival of adult *F. candida* was 92%, the mean number of juveniles produced was 114, and the coefficient of variation for number of juveniles was 14%. Results of definitive tests with a reference toxicant, boric acid (positive control) are shown in Figure 26 and discussed in Section 4.3.1.3. Compliance with the test validity criteria confirmed that the toxicological effects determined in the definitive tests were attributable to the NG treatments.

Ecotoxicological responses of *F. candida* to NG weathered-and-aged in SSL2007d soil are shown in Table 56. Both juvenile production and adult survival were affected in NG-amended soil within the concentration range selected for the definitive test (Figure 50). Exposure to the NG soil treatments significantly (p<0.05) reduced both production of juveniles and adult survival, compared with respective values for the carrier control. The bounded NOEC and LOEC values for production of juveniles were 0.2 and 0.6 mg/kg, respectively. The bounded NOEC and LOEC values for adult survival were 0.2 and 0.6 mg/kg, respectively. Nonlinear regression analyses of toxicity data yielded the respective EC_{20} and EC_{50} values (and corresponding 95 percent CI), mg/kg, of 2 (0.9-3) and 9 (6-12) for adult survival (Gompertz model), and of 1.3 (0.5-2.1) and 6 (3-9) for juvenile production (Gompertz model).

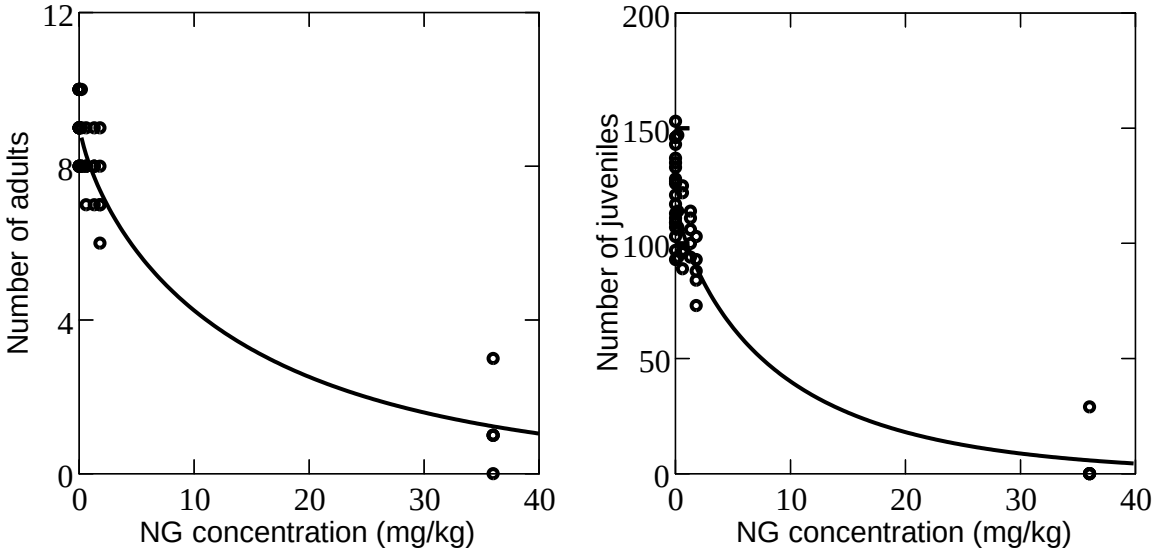


Figure 50. Effect of NG weathered-and-aged in Sassafras sandy loam soil on adult survival (left) and production of juveniles (right) by *Folsomia candida.*

Table 56. Summary of toxicological parameters for NG weathered-and-aged in Sassafras sandy loam soil determined in the definitive toxicity test with *Folsomia candida*.

Ecotoxicological parameters	NG (mg/kg)
Adult survival	
NOEC	0.2
p	0.215
LOEC	0.6
p	0.012
EC_{20}	2
Confidence intervals (95%)	0.9-3.0
EC_{50}	9
Confidence intervals (95%)	6-12
Model used	Gompertz
R^2	0.990
Production of juveniles	
NOEC	0.2
p	0.26
LOEC	0.6
p	0.04
EC_{20}	1.3
Confidence intervals (95%)	0.5-2.1
EC_{50}	6
Confidence intervals (95%)	3-9
Model used	Gompertz
R^2	0.980

Table notes: Toxicity benchmarks are based on analytically determined chemical concentrations (USEPA Method 8330A). EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration.

6.7. Discussion: Effects of energetic materials on soil invertebrates

This portion of the project was undertaken to produce scientifically-defensible toxicity data for the development of soil invertebrate-based Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG, and to investigate and characterize predominant soil physico-chemical parameters that can affect the bioavailability and resulting toxicity of 2,4-DNT to soil invertebrates. To achieve the first objective, studies were designed to meet specific USEPA criteria (USEPA, 2005). Eco-SSL test acceptance criteria were met or exceeded in these investigations by ensuring that: (1) tests were conducted in natural soils having physico-chemical characteristics that support high relative bioavailability of the EM compounds tested; (2) experimental designs for laboratory studies were documented and appropriate; (3) both nominal

and analytically determined concentrations of chemicals of interest were reported; (4) tests included both negative and positive controls; (5) chronic or life cycle tests were used; (6) appropriate chemical dosing procedures were reported; (7) concentration-response relationships were reported; (8) statistical tests used to calculate the benchmark and level of significance were described; and (9) the origin of test species was specified and appropriate.

Definitive studies using soil invertebrates exposures in TSL or SSL soils established new ecotoxicological data for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG effects on soil invertebrates under conditions of very high relative bioavailability for organic chemicals in soil (as defined in USEPA, 2005). The present studies also confirmed the toxicity benchmarks established for 2,4-DNT in our previous studies with SSL soil (Kuperman *et al.*, 2006b; 2004b; Simini *et al.*, 2006). Toxicological benchmarks for 2,4-DNT, and HMX, established in the present studies were generally consistent with data for soil invertebrates reported in a comprehensive review by Kuperman *et al.* (2009). Acute toxicities of 2-ADNT, and 4-ADNT determined in our studies with *E. fetida* comport with results of Lachance *et al.* (2004) obtained in studies with the earthworm *E. andrei*.

The juvenile production endpoints used in the present studies were more sensitive measures of EM toxicity to earthworms and potworms in all soils tested compared with the adult survival endpoint. This comports with results reported in literature for earthworms (Phillips *et al.*, 1993; Robidoux *et al.*, 2002a; 2001b; 2000; Simini *et al.*, 2006; 2003), and potworms (Dodard *et al.*, 2005; 2003; Schafer, 2002; Schafer and Achazi, 1999; Kuperman *et al.*, 2006b; 2006c; 2005; 2004a; 2004b; 2003a; 1999). These findings supported the Eco-SSL requirement of using reproduction endpoints for toxicity benchmark development (USEPA, 2005). Overall, the present definitive studies using *E. fetida*, *E. crypticus*, and *F. candida* exposures in TSL or SSL soils established ecotoxicological benchmarks for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG in compliance with Eco-SSL test acceptance criteria (USEPA, 2005), thus achieved the first objective of this investigation.

The important role of soil properties in affecting bioavailability and toxicity of energetic soil contaminants to soil invertebrates has been emphasized in several studies (Kuperman *et al.*, 2006b; 2006c; 2005; 2004a; 2003; Schafer, 2002; Simini *et al.*, 2006; 2003; Phillips *et al.*, 1993). To achieve the second objective of the present studies, toxicity testing was conducted with additional natural soils, including KL, and WCL, to extend the range of soil physico-chemical characteristics that were hypothesized to affect 2,4-DNT toxicity to soil invertebrates. The QRB scores for organic chemicals in natural soils were considered “very high” for TSL and SSL and “medium” for KL, and WCL soil, according to the Eco-SSL criteria (USEPA, 2005). Soil-related differences were evident in both acute (adult survival) and chronic (cocoon or juvenile production) toxicity benchmarks established in studies with earthworms or potworms exposed to 2,4-DNT weathered-and-aged in each of the natural soils tested in the present studies. Acute toxicities of 2,4-DNT for *E. fetida* or *E. crypticus* were generally greater in the light textured sandy loam soils compared with the more heavy textured clay loam soils. The order of acute toxicity for *E. fetida* based on the EC_{50} values for 2,4-DNT weathered-and-aged in soil was (from greatest to least toxicity): TSL > KL > WCL. The order of chronic toxicity to *E. fetida* based on the EC_{50} values for 2,4-DNT weathered-and-aged in soil was (from greatest to least toxicity): TSL ≥ SSL ≥ KL > WCL. The order of acute toxicity for *E. crypticus* based on the EC_{50} values for 2,4-DNT weathered-and-aged in soil was (from greatest to least toxicity): SSL ≥ TSL > KL > WCL. The order of chronic toxicity to *E. crypticus* based on the EC_{50} values for

2,4-DNT weathered-and-aged in soil was (from greatest to least toxicity): SSL > TSL > KL > WCL.

The quantitative analyses of relationships among the acute or chronic toxicity benchmarks for 2,4-DNT and soil property measurements revealed that both clay and OM contents of the soil affected toxicity of 2,4-DNT to *E. fetida* or *E. crypticus*. Strong correlations were also detected for several earthworm or potworm adult survival and reproduction endpoints and soil CEC. No significant correlations were found among any toxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter and clay as the dominant properties mitigating 2,4-DNT toxicity for soil annelids (earthworms and potworms). However, no single soil parameter investigated directly explained the variance in toxicity of 2,4-DNT to the collembolan *F. candida.* The specific microenvironment of ecological niches in soil occupied by Collembola (i.e., air-filled soil pores) may minimize their direct contact with chemicals in the soil pore water or within the soil solid phase, compared with the soil annelids which exhibited stronger relationships among toxicity endpoints and soil constituents.

Results of the present studies comport with findings of several published studies that have suggested that bioavailability of 2,4-DNT and related nitroaromatic compounds (NACs) can be affected by the clay content (Emery *et al*., 2001; Haderlein *et al.*, 1996; Singh *et al.*, 2008), OM content (Anzhi *et al.*, 1997; Eriksson and Skyllberg, 2001; Singh *et al.*, 2010), or a combination of the two (Jaenig, 2006). Sorption of NACs to constituents of natural soils is not linearly related with their total concentration in soil, and is dominated by strong and specific interactions with certain matrix components, rather than by hydrophobic partitioning (Monteil-Rivera *et al.*, 2009). Among all matrix components commonly found in soils, including clays, carbonates, quartz, aluminum, iron (hydrous)oxides, and OM, clays were found to be strong sorbents for NACs (Daun *et al.*, 1998; Esteve-Núñez *et al.*, 2001; Haderlein *et al.*, 1996; Weissmahr *et al.*, 1997; 1998). NACs can be sorbed to uncharged regions of phyllosilicate clays through electron donor-acceptor complexes between oxygen atoms of the siloxane surface and the six-carbon ring through pi-bonding (Haderlein *et al.*, 1996; Weissmahr *et al.*, 1998). Consequently, the adsorption of NACs can be strongly affected by exchangeable cations (Haderlein *et al.*, 1996), which may partially explain significant correlation found in the present studies between the toxicity benchmarks for 2,4-DNT and soil CEC. In aqueous environments, adsorption of the NACs to clays is high when the exchangeable cations at the clays are a mixture that includes K^+ and NH_4^+ but is negligible for homoionic Na^+-, Ca^{2+}-, Mg^{2+}-, and Al^{3+}-clays (Haderlein *et al.*, 1996; Weissmahr *et al.*, 1997). Furthermore, the affinity and the adsorption capacity of the clays for NACs increase in the order kaolinite < illite < montmorillonite. Thus, clay minerals, plus their abundance and degree of K^+- and NH_4^+-saturation, can control the phase distribution and bioavailability of NACs in soils (Haderlein *et al.*, 1996). NACs and their metabolites were also shown to react and sorb to OM in the soil (Achtnich *et al.*, 1999; Anzhi *et al.*, 1997; Dawel *et al.*, 1997; Drzyzga *et al.*, 1998; Eriksson and Skyllberg, 2001; Esteve-Núñez *et al.*, 2001; Simpson, 2006; Singh *et al.*, 2010; Thorn and Kennedy, 2002; Thorn *et al.*, 2002; Xing and Pignatello, 1997; Weiß *et al.*, 2004). Sorption studies with low-polarity organic compounds, including nitroaromatic energetic materials have shown that binding of these compounds to both soil OM (Xing and Pignatello, 1997) and silicate clays (Haderlein *et al.*, 1996) is competitive, selective, non-linear, and frequently reversible. Overall, the aforementioned mechanisms affecting the fate of 2,4-DNT in soil are consistent with findings of the present studies that identified soil organic matter and clay as the dominant properties mitigating 2,4-DNT toxicity for soil annelids.

7. Effects of energetic materials on biologically-mediated processes in SSL soil

Assessment and protection of the terrestrial environment at defense installations can be advanced by developing and applying scientifically based ecotoxicological benchmarks. Such benchmarks can help to identify concentrations of contaminant EM in soil that don't present an unacceptable ecological risk for biologically-mediated processes in soil, so managers can better focus remediation resources on those that do. We conducted this research to establish ecotoxicological data that are acceptable for developing such benchmarks for 2,4-DNT, 2-ADNT, 4-ADNT, and NG for use in scientifically based ERA.

7.1. Effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on litter decomposition in SSL soil

Exposure to 2,4-DNT in SSL significantly (p=0.032) inhibited (greater percent of mass remaining) litter decomposition in the 9343 mg/kg treatment after one month compared with carrier control (Figure 51). Inhibition in this treatment remained significant ($p\leq$0.001) throughout the eight-month study. Litter decomposition was also significantly ($p\leq$0.003) inhibited in the 1274 mg/kg treatment after six and eight months compared with carrier control (Figure 51), thus indirectly suggesting an adverse effect on microbial activity at these 2,4-DNT concentrations in soil. Decomposition was significantly (p<0.015) stimulated in the 3.8 and 62 mg/kg treatments after four and six months compared with carrier control. However, this effect was transient and percent of mass remaining in each of these treatments was not significantly ($p\geq$0.507) different from carrier control by the end of the eight-month study (Figure 51).

There was a transient stimulatory effect of 2-ADNT on litter decomposition after the four-month exposure (Figure 52). Litter decomposition was significantly ($p\leq$0.042) increased in the ≥117 mg/kg 2-ADNT treatments compared with carrier control after two and four months, and remained significantly (p=0.001) greater in the 10000 mg/kg treatment after six months (Figure 53). However, percent of mass remaining in any of 2-ADNT treatments was not statistically ($p\geq$0.279) different compared with carrier by the end of the eight-month study (Figure 52). Litter decomposition was not significantly ($p\geq$0.10) different among any of the 4-ADNT treatments throughout the eight-month study (Figure 53).

Exposure to NG significantly ($p\leq$0.001) inhibited litter decomposition in the ≥950 mg/kg treatments compared with carrier control during the eight-month study (Figure 54). Litter decomposition in the 29 mg/kg treatment was not significantly ($p\geq$0.134) different from carrier control during the same exposure period (Figure 54).

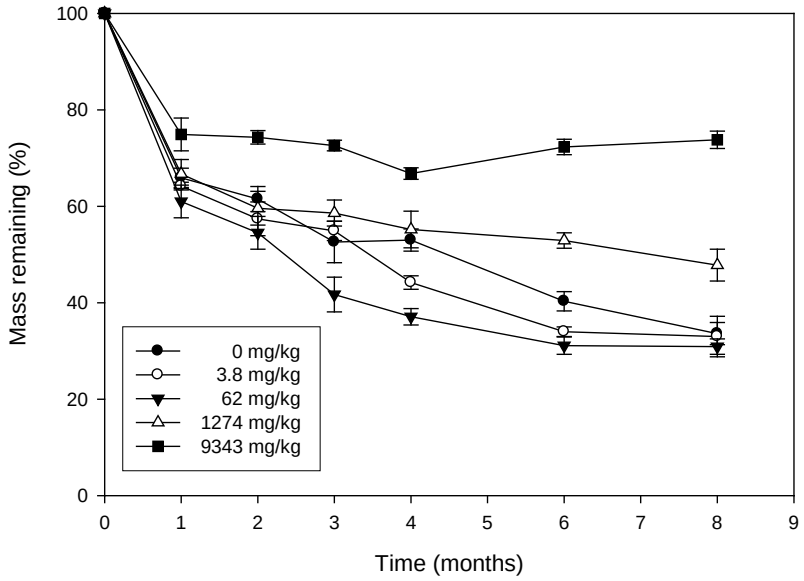


Figure 51. Effect of 2,4-DNT on decomposition of Orchard grass (*Dactylis glomerata)* litter in Sassafras sandy loam soil. Values are means ± SE (n=4).

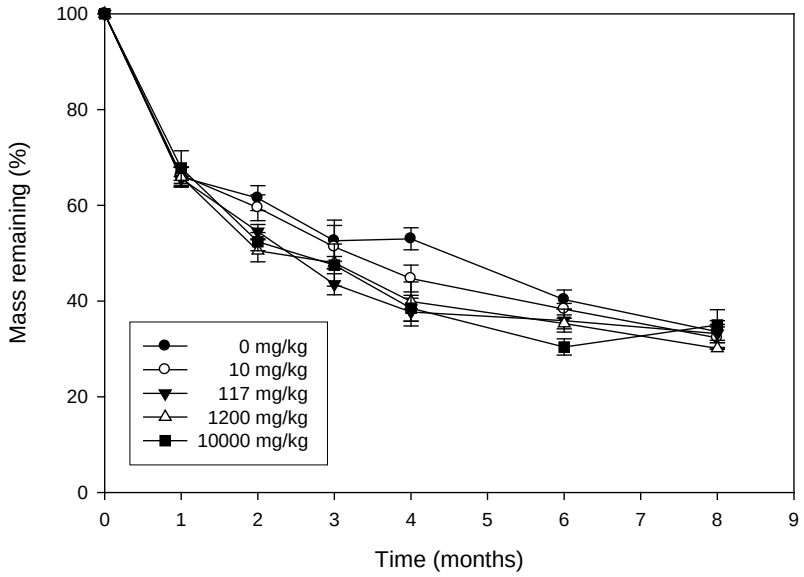


Figure 52. Effect of 2-ADNT on decomposition of Orchard grass (*Dactylis glomerata)* litter in Sassafras sandy loam soil. Values are means ± SE (n=4).

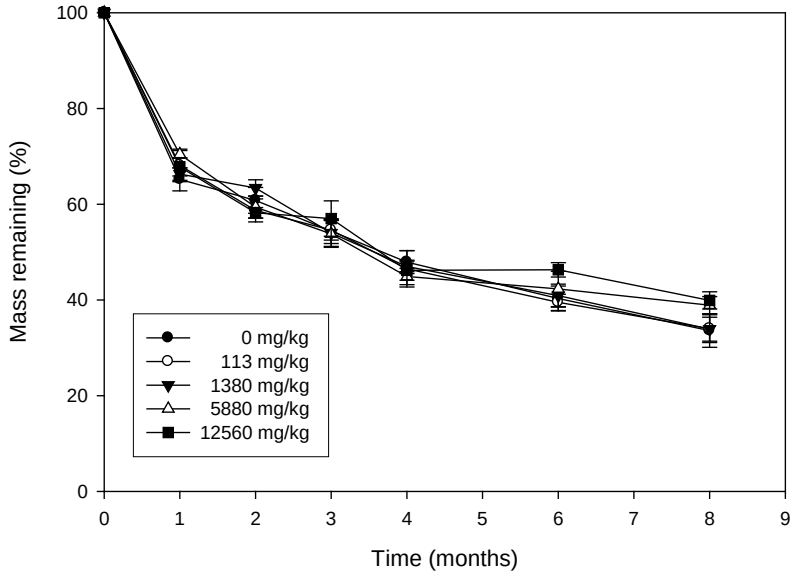


Figure 53. Effect of 4-ADNT on decomposition of Orchard grass (*Dactylis glomerata)* litter in Sassafras sandy loam soil. Values are means ± SE (n=4).

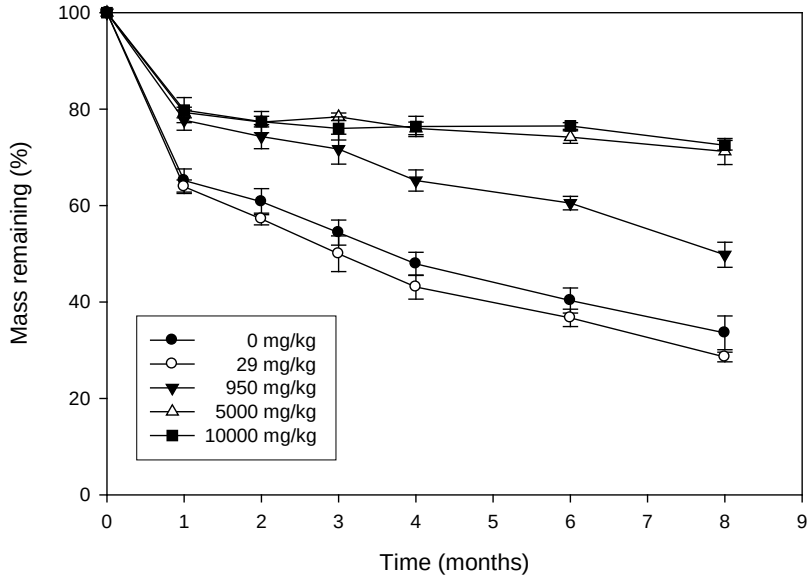


Figure 54. Effect of NG on decomposition of Orchard grass (*Dactylis glomerata)* litter in Sassafras sandy loam soil. Values are means ± SE (n=4).

The ranges of 2,4-DNT and NG concentrations selected for these studies were sufficient to establish the concentration-response relationship for the effects of respective EM on litter decomposition over the entire eight-month study (Figures 55 and 56) based on annual decomposition rate constants (*k*) for litter residues shown in Table 57.

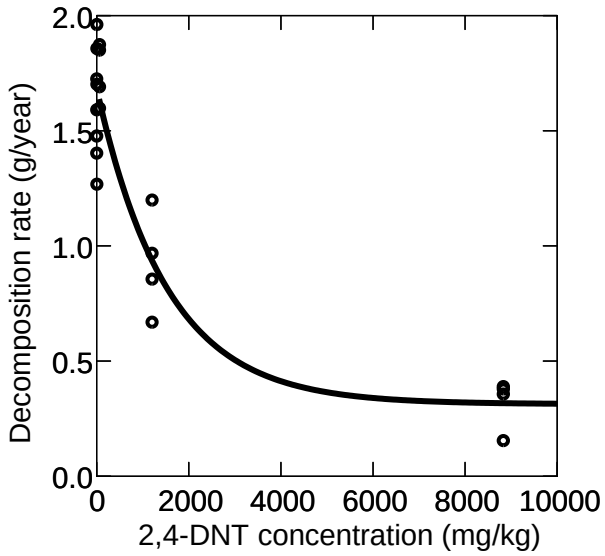


Figure 55. Effect of 2,4-DNT on annual decomposition rate of Orchard grass (*Dactylis glomerata)* litter in Sassafras sandy loam soil.

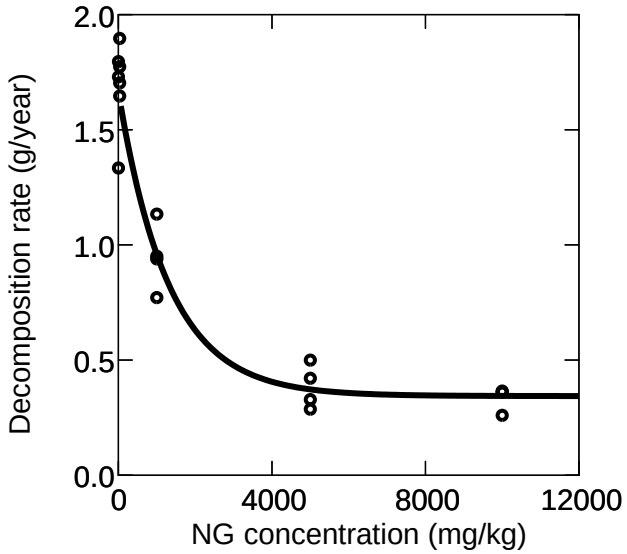


Figure 56. Effect of NG on annual decomposition rate of Orchard grass (*Dactylis glomerata)* litter in Sassafras sandy loam soil.

Nonlinear regression analyses of annual decomposition rate data yielded the EC_{20} and EC_{50} values (mg/kg) of 361 and 1122, respectively, for 2,4-DNT; and 277 and 860, respectively, for NG. Annual decomposition rate constants were not significantly affected by any of 2-ADNT ($p \geq 0.153$) or 4-ADNT ($p \geq 0.065$) treatments (Table 58). Table 59 summarizes ecotoxicological benchmarks for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on litter decomposition.

Table 57. Decomposition rate parameters for Orchard grass (*Dactylis glomerata)* litter exposed to 2,4-DNT or NG in SSL soil for eight months.

2,4-DNT† (mg/kg)	*k*	r^2	NG† (mg/kg)	*k*	r^2
0	-1.550 ± 0.216[a]	0.911	0	-1.547 ± 0.221[a]	0.908
3.8	-1.696 ± 0.259[a]	0.895	29	-1.755 ± 0.243[a]	0.913
62	-1.753 ± 0.377[a]	0.813	950	-0.948 ± 0.125[b]	0.919
1274	-0.922 ± 0.271[b]	0.698	5000	-0.383 ± 0.143[c]	0.590
9343	-0.320 ± 0.219[c]	0.298	10000	-0.337 ± 0.152[c]	0.496

Table notes: †Values are soil concentrations determined by USEPA Method 8330A; Annual decomposition rate constants (*k*) and coefficients of determination (r^2) are based on a single negative exponential model; *k* values are means ± standard errors (n=4); *k* values for each energetic material with the same letter are not significantly different (ANOVA and FLSD test at $p \leq 0.05$ level).

Table 58. Decomposition rate parameters for Orchard grass (*Dactylis glomerata)* litter exposed to 2-ADNT or 2-ADNT in SSL soil for eight months.

2-ADNT† (mg/kg)	*k*	r^2	4-ADNT† (mg/kg)	*k*	r^2
0	-1.550 ± 0.216	0.911	0	-1.547 ± 0.221	0.908
10	-1.644 ± 0.237	0.906	113	-1.557 ± 0.230	0.902
117	-1.625 ± 0.367	0.797	1380	-1.546 ± 0.218	0.909
1200	-1.723 ± 0.312	0.859	5880	-1.366 ± 0.279	0.828
10000	-1.689 ± 0.389	0.790	12560	-1.238 ± 0.279	0.798

Table notes: †Values are soil concentrations determined by USEPA Method 8330A; Annual decomposition rate constants (*k*) and coefficients of determination (r^2) are based on a single negative exponential model; *k* values are means ± standard errors (n=4). *k* values were not significantly different among any treatments of each energetic material (ANOVA and FLSD test at $p \leq 0.05$ level).

Table 59. Summary of toxicity benchmarks for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on decomposition of Orchard grass (*Dactylis glomerata)* litter in Sassafras sandy loam soil.

Ecotoxicological parameters	2,4-DNT[‡] mg/kg	2-ADNT mg/kg	4-ADNT mg/kg	NG[‡] mg/kg
NOEC	62	10	12560[††]	29
p	0.161	0.073	0.280	0.064
LOEC	1274	117[†]	>12560	950
p	<0.0001	0.003		<0.0001
EC_{20} (95% CI)	361 (168-554)	>10000	>12560	277 (158-395)
EC_{50} (95% CI)	1122 (523-1721)	>10000	>12560	860 (492-1228)
R^2	0.981	ND	ND	0.984

Table notes: Values are soil concentration determined by USEPA Method 8330A. EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration. [‡]Values determined on the basis of annual decomposition rate constants; [†]A transient effect based on ANOVA and FLSD test of percent mass remaining after a four-month exposure; No significant difference in mass loss (p>0.05) among any 2-ADNT concentrations at the end of the eight-month study; [††]Unbounded NOEC based on ANOVA and FLSD test of percent mass remaining during the eight-month exposure; ND=Not Determined.

7.2. Effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on soil respiration

Separate batches of SSL2011 soil were individually amended at ECBC with 2,4-DNT, 2-ADNT, and 4-ADNT to achieve nominal concentrations of 0 (carrier control), 10, 100, 1000, and 10000 mg/kg dry soil for use in respective basal respiration assays and substrate-induced respiration assays. Nominal and analytically determined concentrations of each EM at the beginning of each soil respiration assay are presented in Table 60. Mean values for 2,4-DNT within freshly amended soil treatments used in basal respiration assay, expressed as percentage of amendment, ranged from 90% at nominal 100 mg/kg to 145% at nominal 10000 mg/kg (Table 60). These values ranged from 85% at nominal 100 mg/kg to 120% at nominal 10 mg/kg for 2-ADNT, and from 59% at nominal 100 mg/kg to 97% at nominal 1000 mg/kg for 4-ADNT (Table 60).

Mean values for 2,4-DNT within freshly amended soil treatments used in substrate-induced respiration assay, expressed as percentage of amendment, ranged from 60% at nominal 100 mg/kg to 91% at nominal 10 mg/kg (Table 60). These values ranged from 49% at nominal 1000 mg/kg to 170% at nominal 10 mg/kg for 2-ADNT; and from 62% at nominal 10 mg/kg to 110% at nominal 1000 mg/kg for 4-ADNT (Table 60). The recoveries of DNTs were confirmed by using an internal standard (1,3-DNB in subsamples of the 100 mg/kg and 5000 mg/kg treatments). The average (n=3) recovery of 1,3-DNB ranged from 87 to 104%. No 2,4-DNT or

ADNTs were detected in carrier controls above the analytical detection limits of 0.1 mg/kg and 0.05 mg/kg, respectively.

Table 60. Nominal and analytically determined concentrations of 2,4-DNT, 2-ADNT, and 4-ADNT in Sassafras sandy loam (SSL2011) soil used in the 28-day soil respiration study.

Nominal concentration	10		100		1000		10000	
	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %	Mean (SD) mg/kg	Recovery %
Assay								
BR 2,4-DNT	9.3 (0.17)	93	90 (5.3)	90	953 (112)	95	14533 (5000)	145
BR 2-ADNT	12 (6.3)	120	85 (5.4)	85	1011 (55)	101	11542 (4364)	115
BR 4-ADNT	9 (3.7)	90	59 (46)	59	973 (107)	97	8104 (438)	81
SIR 2,4-DNT	9.1 (0.31)	91	60 (2.1)	60	904 (81)	90	7708 (1623)	77
SIR 2-ADNT	17 (1.9)	170	91 (17)	91	489 (308)	49	6079 (4578)	61
SIR 4-ADNT	6.2 (2.04)	62	86 (8.5)	86	1097 (184)	110	7819 (85)	78

Table notes: Values are soil concentration means (n=3) and Standard Errors (SD) determined by USEPA Method 8330A. BR=basal respiration; SIR=substrate-induced respiration. No 2,4-DNT or ADNTs were detected in carrier controls above the analytical detection limits of 0.1 mg/kg and 0.05 mg/kg, respectively.

Separate batches of SSL2011 soil were individually amended at General Dynamics with NG to achieve nominal concentrations of 0 (carrier control), 10, 100, 500, and 1000 mg/kg dry soil for use in basal respiration assay, and 0 (carrier control), 10, 100, 1000, and 5000 mg/kg dry soil for use in substrate-induced respiration assay. Nominal and analytically determined concentrations of NG at the beginning of each soil respiration assay are presented in Table 61. Mean values for NG within freshly amended soil treatments used in basal respiration assay, expressed as percentage of amendment, ranged from 51% at nominal 100 mg/kg to 82% at nominal 10 mg/kg (Table 61). Mean values for NG within freshly amended soil treatments used in substrate-induced respiration assay ranged from 47% at nominal 100 mg/kg to 82% at nominal 10 mg/kg (Table 61). The recovery of NG was confirmed by using an internal standard (1,3-DNB in subsamples of the 100 mg/kg and 5000 mg/kg treatments). The average (n=3) recovery of 1,3-DNB ranged from 97 to 104%. No NG or its degradation products were detected in carrier controls above the analytical detection limit (0.5 mg/kg).

Table 61. Nominal and analytically determined concentrations of NG in Sassafras sandy loam (SSL2011) soil used in the 28-day soil respiration study.

Nominal Concentration	Mean mg/kg	SD	Recovery %
Basal respiration			
10	8.7	0.61	87
100	51	2	51
500	346	8	69
1000	687	54	69
Substrate-induced respiration			
10	8.2	0.26	82
100	47	3	47
1000	665	93	66
5000	3521	385	70

Table notes: Values are soil concentration means (n=3) and Standard Errors (SD) determined by USEPA Method 8330A. No NG or its degradation products were detected in carrier controls above the analytical detection limit (0.5 mg/kg).

The presence of a contaminant can affect biological activity in soil and therefore alter CO_2 production measured by the respiration rate. The ranges of 2,4-DNT, 2-ADNT, and 4-ADNT concentrations selected for the present studies were sufficient to establish the concentration-response relationship for the effects of respective EM on BR after 28 days of incubation (Figures 57, 58, and 59). Exposure to 2,4-DNT for 28 days significantly (p<0.0001) inhibited BR at 90 mg/kg (LOEC), compared to BR in the carrier control. The exponential model had the best fit (R^2=0.997) for BR data on day 28 (Figure 57), yielding the EC_{20} and EC_{50} values (mg/kg) of 651 and 2023, respectively (Table 62). The BR rate was significantly inhibited (compared with carrier control) at the lowest positive concentrations of either 2-ADNT or 4-ADNT, producing the respective unbounded LOEC values (mg/kg) of 12 and 9 (Table 62). Exponential model had the best fit for BR data for the two EM (R^2=0.998; Table 60), and yielded the EC_{20} and EC_{50} values (mg/kg) of 17 and 54, respectively, for 2-ADNT; and 9 and 28, respectively, for 4-ADNT.

NG was the least toxic for BR among the four EM tested in SSL soil. Concentration range selected in the present study was sufficient to establish the concentration-response relationship for the effects of NG on BR after 28 days of incubation in SSL soil (Figure 60). The logistic Gompertz model had the best fit (R^2=0.988) for BR data yielding the EC_{20} and EC_{50} values (mg/kg) of 163 and 342, respectively (Table 62). Table 62 summarizes ecotoxicological benchmarks for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on BR in SSL soil.

Table 62. Toxicity benchmarks for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on basal respiration in Sassafras sandy loam soil determined in the 28-day study.

Ecotoxicological parameters	2,4-DNT mg/kg	2-ADNT mg/kg	4-ADNT mg/kg	NG mg/kg
NOEC	9.3	<12	<90	51
p	0.314	ND	ND	0.765
LOEC	90	12†	9†	346
p	<0.0001	0.043	0.023	<0.0001
EC_{20} (95% CI)	19 (14-24)	17 (10-25)	9 (5-13)	163 (68-259)
EC_{50} (95% CI)	59 (44-74)	54 (32-77)	28 (16-40)	342 (257-428)
R^2	0.997	0.998	0.998	0.988

Table notes: Soil concentration determined by USEPA Method 8330A. EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration; †Unbounded LOEC; ND=Not Determined: could not be determined within the concentration range tested.

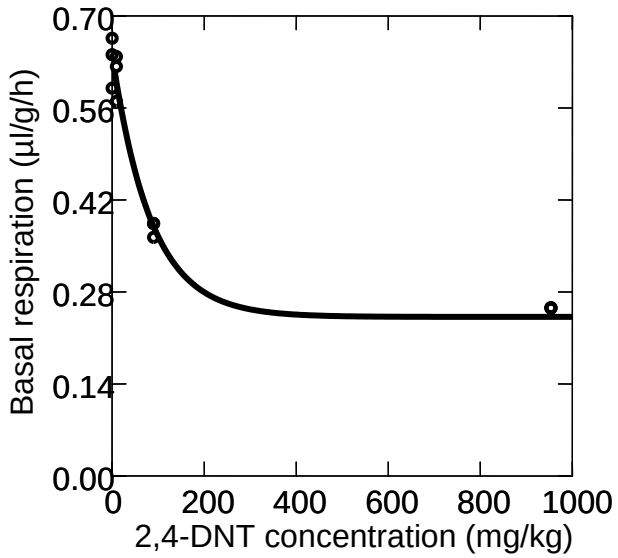


Figure 57. Effect of 2,4-DNT on basal respiration rate in Sassafras sandy loam soil after 28 days.

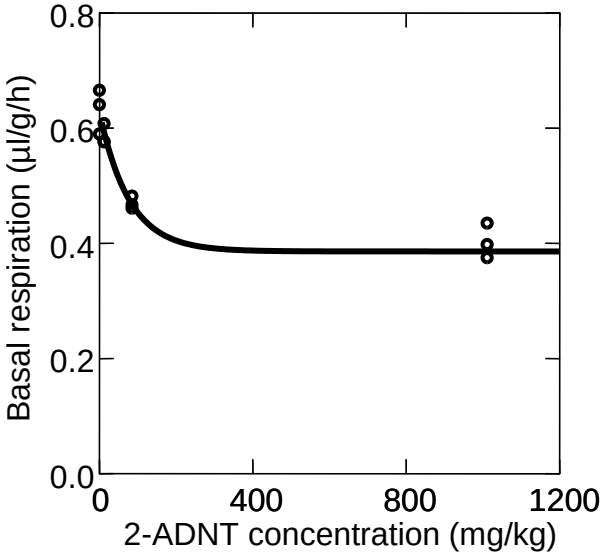


Figure 58. Effect of 2-ADNT on basal respiration rate in Sassafras sandy loam soil after 28 days.

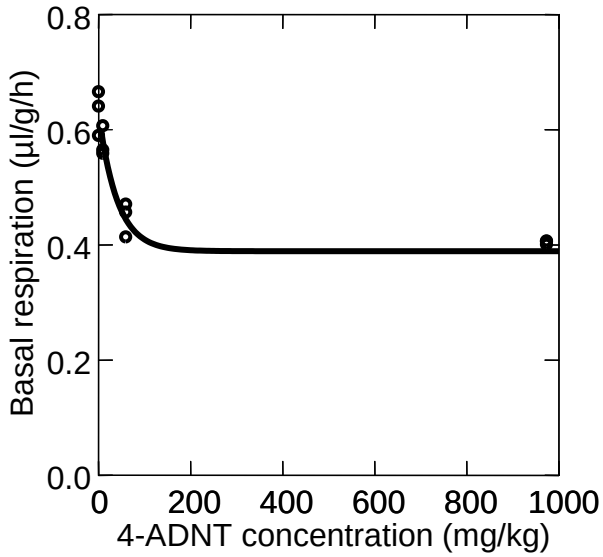


Figure 59. Effect of 4-ADNT on basal respiration rate in Sassafras sandy loam soil after 28 days.

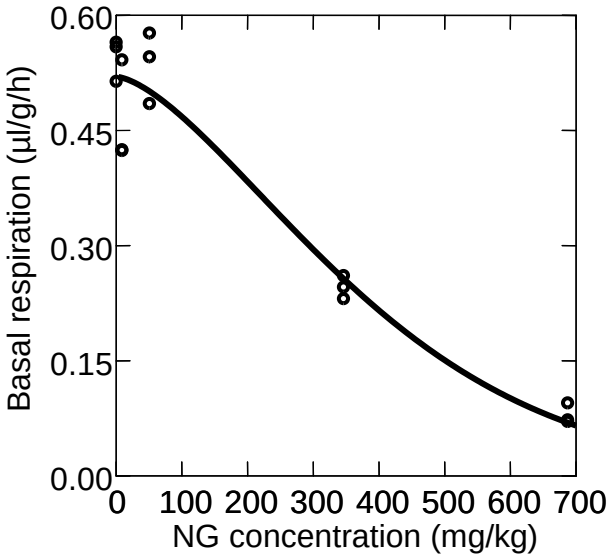


Figure 60. Effect of NG on basal respiration rate in Sassafras sandy loam soil after 28 days.

Amendment of SSL soil with 2,4-DNT significantly (p<0.0001) stimulated SIR in the 60 mg/kg (LOEC) treatment, compared to the carrier control, producing the NOEC of 9 mg/kg and the LOAEC of 904 mg/kg after the 28-d incubation. Consequently, the logistic hormetic model had the best fit (R^2=0.996) for SIR data on day 28 (Figure 61), and yielded the EC_{20} and EC_{50} values (mg/kg) of 878 and 1446, respectively (Table 63).

Exposure to 2,4-DNT significantly (p<0.0001) decreased microbial biomass at 904 mg/kg (LOEC), compared with carrier control, after the 28-d soil incubation (Table 63). The range of 2,4-DNT concentrations selected in this study was sufficient to establish the concentration-response relationship for the effects of 2,4-DNT on microbial biomass C (Figure 61). The logistic hormetic model had the best fit for the biomass C data (R^2=0.992) and yielded the EC_{20} and EC_{50} values (mg/kg) of 105 and 325, respectively (Table 63).

Soil amendment with either 2-ADNT or 4-ADNT significantly ($p\leq$0.001) stimulated SIR in the 91 mg/kg and 86 mg/kg (the LOEC values) treatments, respectively, compared to the carrier control. No inhibition of SIR was found up to and including the greatest concentrations of each EM tested in SSL, producing the corresponding unbounded NOAEC values of 6078 mg/kg for 2-ADNT, and 7819 mg/kg for 4-ADNT, after the 28-d incubation.

Microbial biomass C was significantly (p=0.003) decreased at 2-ADNT concentration of 489 mg/kg (LOEC), compared with carrier control, after the 28-d soil incubation (Table 63). However, no concentration-response relationship could be determined for the effects of 2-ADNT on biomass C within the range of concentrations tested in SSL2011 soil. Consequently, Maximum Allowable Toxic Concentration (MATC) was determined for the effect of 2-ADNT on biomass C. MATC was calculated as geometric mean of the NOEC and LOEC values shown in Table 63, and is an accepted alternative to EC_{20} by USEPA (2005). Soil amendment with 4-ADNT significantly (p=0.005) decreased biomass C at 1097 mg/kg (LOEC). The range of 4-

ADNT concentrations selected in this study was sufficient to establish the concentration-response relationship for the effects of 4-ADNT on microbial biomass C for the 20% effect level. The logistic Gompertz model had the best fit for the biomass C data (R^2=0.992) and yielded the EC_{20} value of 1663 mg/kg (Table 63). Estimate of the 50% effect level (EC_{50} value) was outside the range of 4-ADNT concentrations tested in the present study.

Amendment of SSL soil with NG significantly (p=0.020) inhibited SIR in the 47 mg/kg (LOEC) treatment, compared to the carrier control, after the 28-d incubation (Table 63). The range of NG concentrations selected in this study was sufficient to establish the concentration-response relationship for the effects of NG on SIR (Figure 62). Exponential model had the best fit for SIR data (R^2=0.985; Table 63), and yielded the EC_{20} and EC_{50} values (mg/kg) of 25 and 77, respectively (Table 63).

Exposure to NG significantly (p<0.0001) decreased microbial biomass at 47 mg/kg (LOEC), compared with carrier control, after the 28-d soil incubation (Table 63). The range of NG concentrations selected in this study was sufficient to establish the concentration-response relationship for the effects of NG on microbial biomass C (Figure 62). Exponential model had the best fit for the biomass C data (R^2=0.994), and yielded the EC_{20} and EC_{50} values (mg/kg) of 24 and 75, respectively (Table 63).

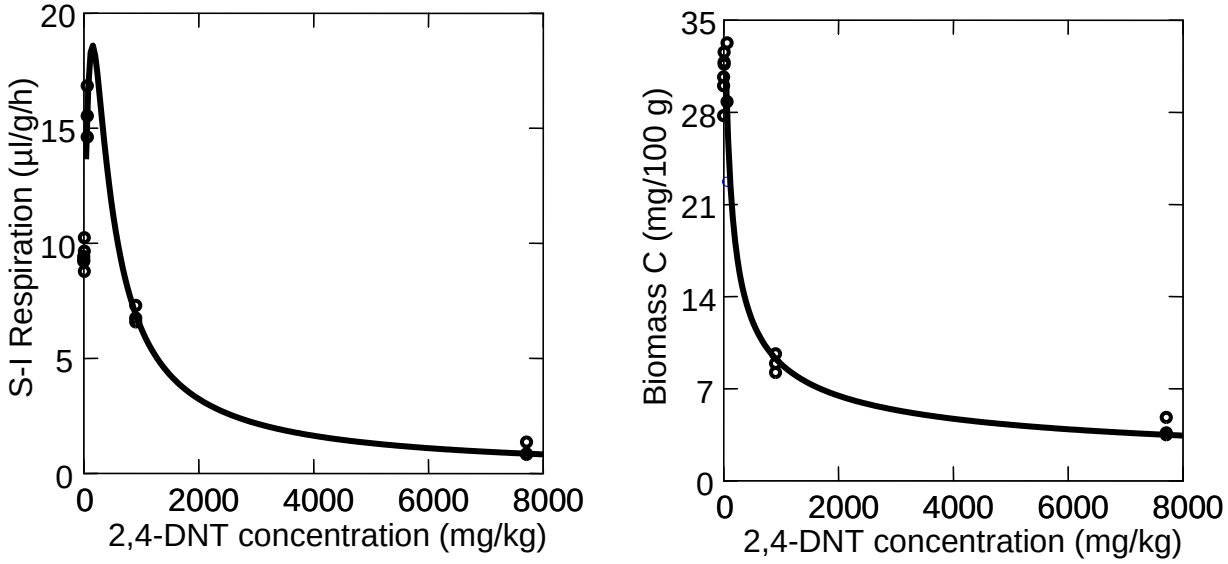


Figure 61. Effect of 2,4-DNT on substrate-induced respiration (left) and microbial biomass carbon (right) in Sassafras sandy loam soil after 28 days.

Table 63 summarizes ecotoxicological benchmarks for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on SIR, and microbial biomass C, in SSL2011 soil. The results of present studies indicate that these EM can negatively impact soil respiration and microbial biomass in SSL soil even in the presence of a carbon energy source.

Table 63. Toxicity benchmarks for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on substrate-induced respiration (SIR) and microbial biomass carbon (Biomass C) in Sassafras sandy loam soil determined in the 28-day study.

Ecotoxicological Parameter	2,4-DNT mg/kg	2-ADNT mg/kg	4-ADNT mg/kg	NG mg/kg
SIR				
NOEC	9	17	6.2	8.2
p	0.642	0.247	0.286	0.887
LOEC	60[†]	91[†]	86[†]	47
p	<0.0001	0.001	<0.0001	0.020
EC_{20} (95% CI)	878 (735-1021)	ND	ND	25 (5-44)
EC_{50} (95% CI)	1446 (1048-1845)	ND	ND	77 (17-138)
R^2	0.996	ND	ND	0.985
Biomass C				
NOEC	60	91	86	8.2
p	0.569	0.584	0.179	0.504
LOEC	904	489	1097	47
p	<0.0001	0.003	0.005	<0.0001
EC_{20} (95% CI)	105 (59-150)	211[††]	1663 (0-3536)	24 (13-36)
EC_{50} (95% CI)	325 (181-471)	ND	NA	75 (39-112)
R^2	0.992	ND	0.997	0.994

Table notes: Soil concentration determined by USEPA Method 8330A. EC=effect concentration.
[†]LOEC based on stimulation of SIR; LOAEC =904 mg/kg for 2,4-DNT; corresponding unbounded NOAEC values are 6078 mg/kg for 2-ADNT and 7819 mg/kg for 4-ADNT.
[††]MATC (Geometric Mean of NOEC and LOEC values). NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration; NOAEC=no-observed-adverse-effect concentration (statistically significant stimulation); LOAEC=lowest-observed-adverse-effect concentration. ND=Not Determined – could not be determined within the concentration range tested. NA=Not Applicable – estimate outside the range of tested concentrations.

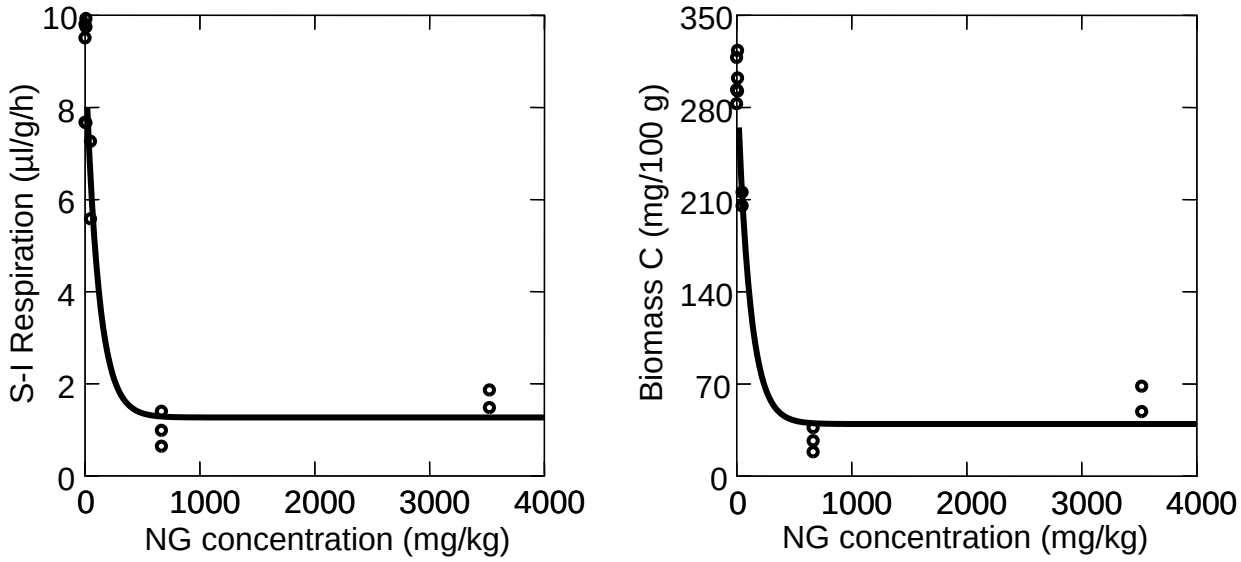


Figure 62. Effect of NG on substrate-induced respiration (left) and microbial biomass carbon (right) in Sassafras sandy loam soil after 28 days.

7.3. Effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on enzymatic activities in SSL soil

Studies were performed to determine the activity levels of soil enzymes in SSL2007e soil freshly amended with individual EM concentrations. Positive nominal/analytically determined EM concentrations at commencement of enzymatic assays with SSL soil are presented in Tables 9, 13, 16, and 23, and were 10/3.8, 100/62, 1000/1278, and 10000/9343 mg/kg for 2,4-DNT; 10/10, 100/117, 1000/1200, and 10000/10000 mg/kg for 2-ADNT; and 100/113, 1000/1380, 5000/5880, and 10000/12560 mg/kg for 4-ADNT. For NG, two sets of amendments were done, and these respective concentrations were 100/64, 1000/974, 5000/5775, and 10000/9785 mg/kg for the APA, NAG, and DH assays; and 10/2.7, 100/61, 1000/990, 5000/4639, and 10000/8929 mg/kg for PN assays.

2,4-DNT significantly ($p \leq 0.021$) inhibited the DH and NAG activities within the concentration range tested, compared to respective carrier controls, but did not affect the AP or PN activities up to and including 9343 mg/kg (unbounded NOEC; Table 64). The range of concentrations selected for these studies was sufficient to establish the concentration-response relationship for the effects of 2,4-DNT on the DH and NAG activities (Figure 63). Exponential model had the best fit for the DH activity data (R^2=0.998), while logistic Gompertz model had the best fit for the NAG activity data (R^2=0.977). Regression analyses of activity data yielded the EC_{20} and EC_{50} values of 16 mg/kg and 50 mg/kg, respectively, for the DH; and the EC_{20} value of 122 mg/kg for NAG. These results revealed that among the enzymatic activites tested, DH activity was the most sensitive endpoint for characterizing the effects of 2,4-DNT on biological activity in SSL soil.

2-ADNT inhibited the AP, DH, and PN activities in a concentration-dependent manner (Figure 64). Logistic hormetic model had the best fit (R^2=0.970) for the AP data due to significant

(p=0.008) stimulation of AP activity in the lowest treatment of 10 mg/kg (LOEC). Logistic Gompertz model had the best fit for the DH activity data (R^2=0.999), while exponential model had the best fit for the PN activity data (R^2=0.999). Regression analyses of the AP, DH and PN activities data yielded the EC_{20} values (mg/kg) of 830, 415 and 175, respectively (Table 64). There was interference in the 10000 mg/kg treatment, from highly colored background that formed, with the measurement of iodo-nitro-tetrazolium formazan used in this assay. Therefore, the DH activity data for the 10000 mg/kg treatment were excluded from regression analysis, and are not shown in Figure 64. The NAG activity was significantly (p=0.044) inhibited by 2-ADNT only in the 1200 mg/kg treatment, compared with carrier control, thus allowing determination of the Maximum Allowable Toxic Concentration (MATC, geometric mean of the NOEC and LOEC values) of 375 mg/kg (Table 64). The PN was the most sensitive among the four enzymes tested for assessing the effects of 2-ADNT on enzymatic activity in SSL soil.

4-ADNT inhibited the AP, DH, and PN activities within the concentration range tested, but did not affect the NAG activity up to and including 12560 mg/kg (unbounded NOEC; Table 64). Interferences, similar to those observed in the studies with 2-ADNT, occurred in some replicates of 5880 and 12560 mg/kg treatments. Therefore, regressions were conducted using data from all replicates of controls, 113 mg/kg, and 1380 mg/kg treatments, and data from a single replicate in 5880 or 12560 mg/kg treatments. The range of concentrations selected for these studies was sufficient to establish the concentration-response relationship for the effects of 4-ADNT on the AP, DH, and PN activities (Figure 65). Exponential model had the best fit for the activities data of the three enzymes (R^2=0.834, 0.991, and 0.997, respectively), and yielded the respective EC_{20} values (mg/kg) of 90, 28, and 113 (Table 64). The DH activity was the most sensitive among the four enzymes tested for assessing the effects of 4-ADNT on enzymatic activity in SSL soil.

NG inhibited the DH activity in a concentration-dependent manner (Figure 66), but did not affect the NAG or PN activities at any concentration tested (NOEC≥9785), compared with carrier control. Exponential model had the best fit for the DH activity data (R^2=0.984). Regression analysis of the DH activity data yielded the EC_{20} value of 34 mg/kg, revealing that DH activity was the most sensitive endpoint for assessing the effects of the four EM tested in these studies on biological activity in SSL soil. The AP activity was significantly (p=0.002) inhibited by NG concentration of 5775 mg/kg (LOEC, Table 64), compared with the carrier control, which allowed determination of the MATC value of 2377 mg/kg.

Table 64. Toxicity benchmarks for the effects of 2,4-DNT, 2-ADNT, 4-ADNT, and NG on enzyme activities in Sassafras sandy loam soil.

Ecotoxicological parameters	2,4-DNT mg/kg	2-ADNT mg/kg	4-ADNT mg/kg	NG mg/kg
AP				
NOEC	9343[‡]	10[†]	113	974
p	0.574	0.008	0.650	0.293
LOEC	>9343	117[††]	1380	5775
p	ND	<0.0001	0.006	0.002
EC_{20}	ND	830	90	2377[†††]
(95% CI)	ND	(0-2400)	(0-335)	ND
R^2	ND	0.970	0.850	ND
DH				
NOEC	<3.8	<10	<113	<64
p	ND	ND	ND	ND
LOEC	3.8[‡‡]	10[‡‡]	113[‡‡]	64[‡‡]
p	0.007	0.001	<0.0001	<0.0001
EC_{20}	16	415	28	34
(95% CI)	(9-23)	(133-697)	(15-42)	(23-45)
R^2	0.998	0.999	0.991	0.984
NAG				
NOEC	<3.8	117	12560	9785[‡]
p	ND	0.157	0.271	0.366
LOEC	3.8[‡‡]	1200	>12560	>9785
p	0.021	0.044	ND	ND
EC_{20}	122	375[†††]	>12560	ND
(95% CI)	(0-1044)	ND	ND	ND
R^2	0.977	ND	ND	ND
PN				
NOEC	9343[‡]	117	<113	8929[‡]
p	0.386	0.197	ND	0.585
LOEC	>9343	1200	113[‡‡]	>8929
p	ND	<0.0001	<0.0001	ND
EC_{20}	ND	175	113	ND
(95% CI)	ND	(74-275)	(84-142)	ND
R^2	ND	0.999	0.997	ND

Table notes: Values are soil concentration determined by USEPA Method 8330A. PN=potential nitrification; DH=dehydrogenase; AP=acid phosphatase; NAG=N-acetyl-glucosaminidase; EC=effect concentration; NOEC=no-observed-effect concentration; LOEC=lowest-observed-effect concentration; [‡]Unbounded NOEC; [‡‡]Unbounded LOEC [†]NOAEC=no-observed-adverse-effect concentration (statistically significant stimulation); [††]LOAEC=lowest-observed-adverse-effect concentration; [†††]MATC= Maximum Allowable Toxic Concentration; ND=Not Determined (no concentration-dependent effect within the concentration range tested).

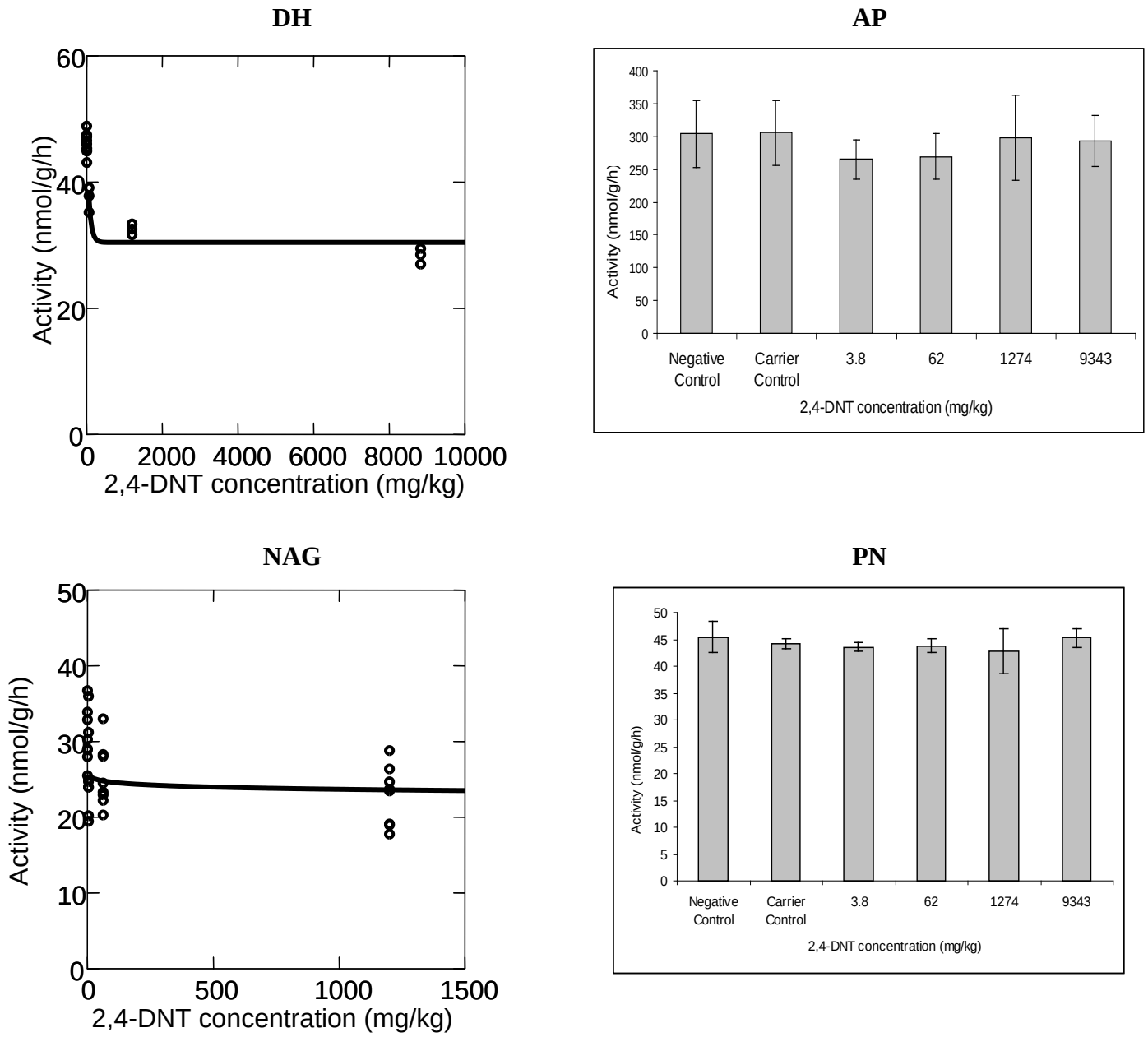


Figure 63. Enzyme activities in Sassafras sandy loam soil amended with 2,4-DNT. Error bars are standard deviations (n=8 for AP and n=3 for PN).

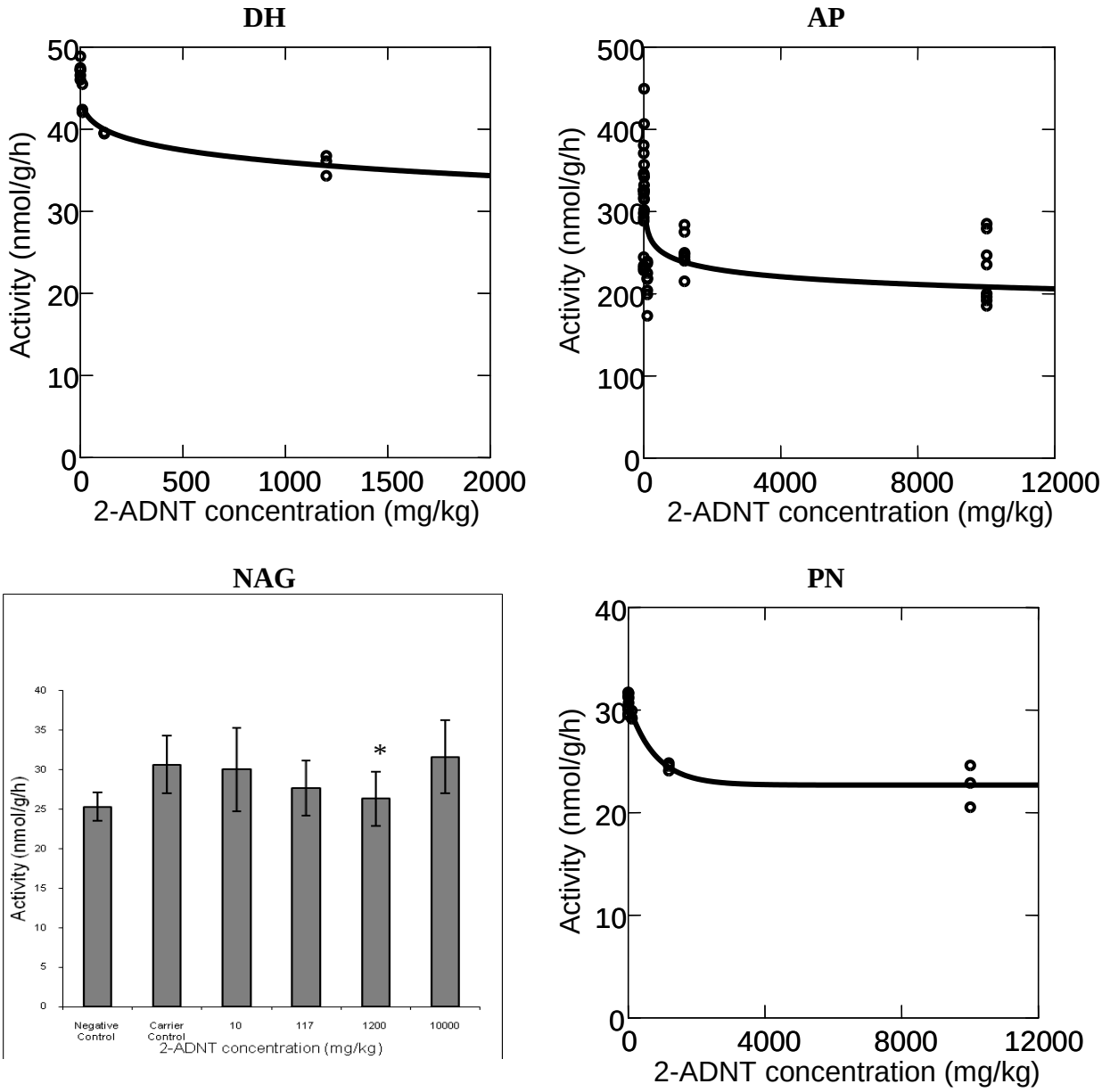


Figure 64. Enzyme activities in Sassafras sandy loam soil amended with 2-ADNT. Error bars are standard deviations (n=8); *Significant (*p*=0.044) inhibition compared with carrier control.

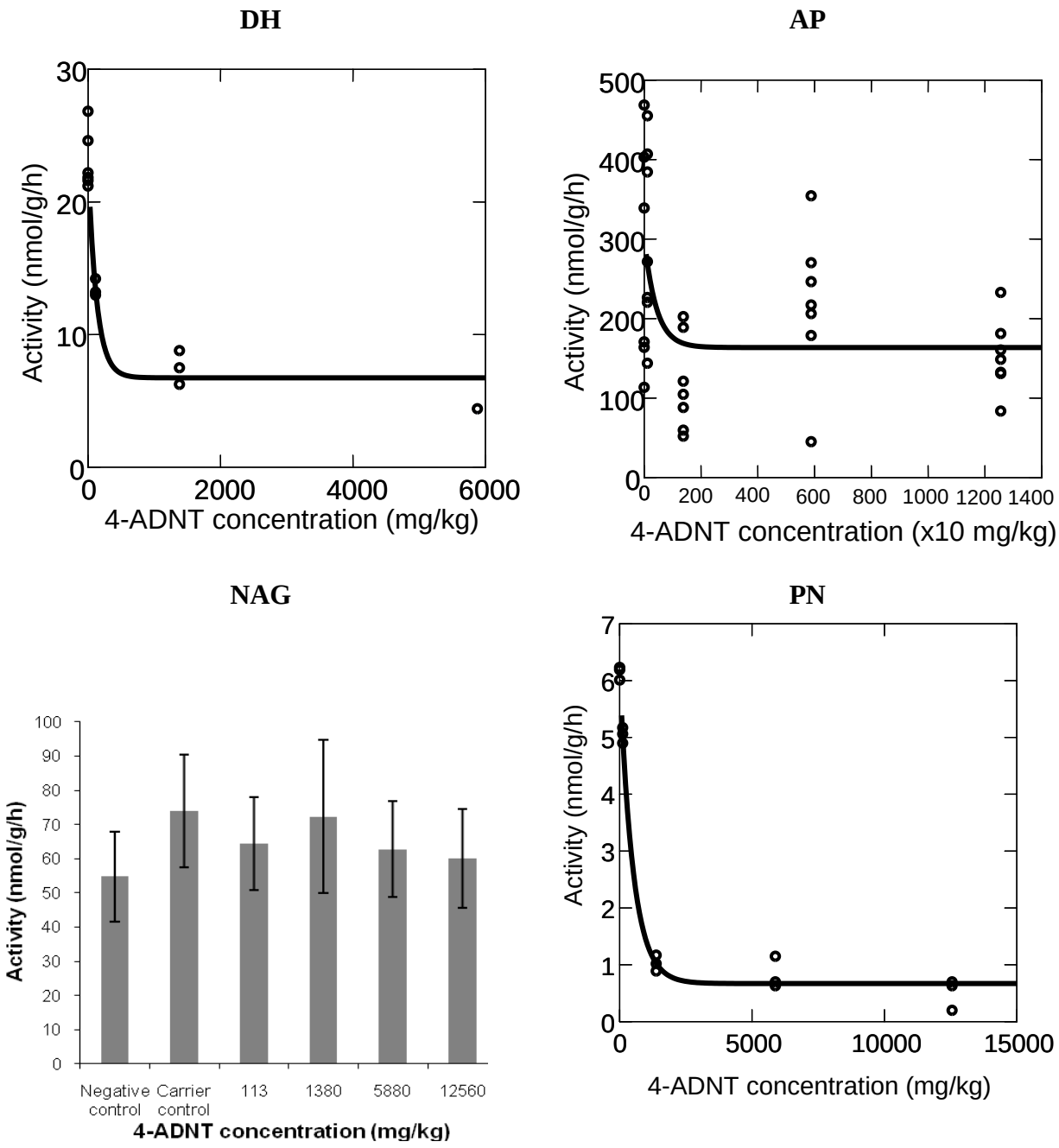


Figure 65. Enzyme activities in Sassafras sandy loam soil amended with 4-ADNT. Error bars are standard deviations (n=8).

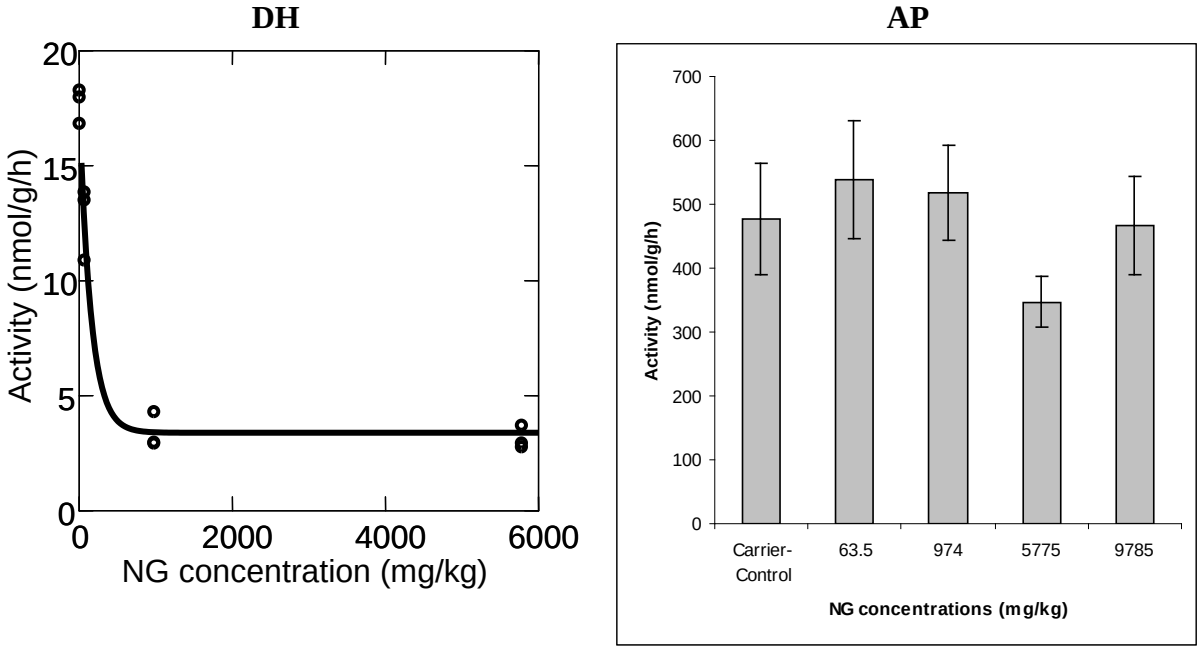


Figure 66. Enzyme activities in Sassafras sandy loam soil amended with NG. Error bars are standard deviations (n=8); *Significant (*p*=0.002) inhibition compared with carrier control.

7.4. Discussion: Effects of energetic materials on soil processes

Maintaining soil quality, fertility, and structure is essential for protecting and sustaining biodiversity and ecological integrity of terrestrial ecosystems. Central to achieving this goal is the need for a greatly improved understanding of the potential effects of EM contaminants on the sustainability of ecosystems at defense installations. Litter decomposition, soil respiration, and soil enzymatic activity are among the most integrating processes within the soil ecosystem because they involve complex interactions of soil microbial, plant, and faunal activities with the soil chemical environment. Any disturbance that alters these biologically-mediated processes can result in nutrient losses and a decline in soil fertility, which can negatively impact sustainability of the environment. Therefore, an assessment of how a release of selected EMs in soil may alter rates of litter decomposition, soil respiration or soil enzymatic activities, and the subsequent rates of nutrient retention and release, is critical to understanding their potential impacts on the overall functioning of the soil ecosystem at military testing and training sites. Various microbial test methods have been developed, validated and standardized by the OECD and ISO (Table 65) in the last 30 years. Toxicological benchmarks developed in the present studies for soil microbial endpoints were based on several of these toxicity assays, including carbon mineralization assays (ISO/14240-1, 1997; ISO/16072, 2002; ISO/17155, 2002; OECD/217, 2000b; USEPA, 1987), organic matter decomposition assay (EC, 2002; OECD, 2006), and soil enzyme activity assays (ISO/14238, 1997, ISO/15685, 2004; ISO/22939, 2010 and ISO/23753-2, 2005b).

Table 65. Standard guidelines for the testing of chemicals on microorganisms published by OECD and ISO.

Test	Title	Year
OECD 216	Soil microorganisms, Nitrogen Transformation Test.	2000
OECD 217	Soil microorganisms, Carbon Transformation Test	2000
OECD GD 56	Guidance document on the breakdown of organic matter in litterbags	2006
ISO 14238	Determination of nitrogen mineralization and nitrification in soils and the influence of chemical on these processes	1997
ISO 14240-1	Determination of soil microbial biomass - Part 1: Substrate-induced respiration method	1997
ISO 14240-2	Determination of soil microbial biomass - Part 2: Fumigation-extraction method	1997
ISO 15685	Determination of potential nitrification and inhibition of nitrification - Rapid test by ammonium oxidation	2004
ISO 16072	Laboratory methods for determination of microbial soil respiration	2002
ISO 17155	Determination of abundance and activity of soil microflora using respiration curves	2002
ISO 18311 (Draft)	Method for testing effects of soil contaminants on the feeding activity of soil dwelling organisms — Bait-lamina test	2012
ISO 22939	Measurement of enzyme activity patterns in soil samples using fluorogenic substrates in micro-well plates	2010
ISO 23753-1	Determination of dehydrogenase activity in soils - Part 1: Method using triphenyltetrazolium chloride (TTC)	2005
ISO 23753-2	Determination of dehydrogenase activity in soils -- Part 2: Method using iodotetrazolium chloride (INT)	2005
ISO 29843-1	Determination of soil microbial diversity - Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis	2010
ISO 29843-2	Determination of soil microbial diversity - Part 2: Method by phospholipid fatty acid analysis (PLFA) using the "simple PLFA extraction method”	2011

Integral to achieving sustainable use of current and future training and testing ranges is the development of environmental quality criteria that can be consistently applied in order to gauge the ecotoxicological impacts of the military operations. Our studies were designed to address immediate critical data needs required for successful management of defense installations in a

sustainable manner, and for the knowledge-based decision making. Assessment and protection of the terrestrial environment at defense installations was advanced by developing and applying scientifically based ecotoxicological benchmarks that can help to identify concentrations of contaminant EM in soil that present an acceptable ecological risk for biologically-mediated processes in soil. Then managers may better focus remediation resources on those EMs that present unacceptable risk. We conducted this research to establish ecotoxicological data that are acceptable for developing such benchmarks for 2,4-DNT, 2-ADNT, 4-ADNT, and NG, for use in scientifically based ERA.

Nitroaromatic EMs introduced into soil during testing and training activities at defense installations can undergo rapid transformation to the amino-nitro intermediates. Frequent co-occurrence of trinitrotoluene (TNT), trinitrobenzene (TNB), DNTs, and ADNTs in soils of contaminated sites or in experimentally contaminated soil treatments have precluded investigators from partitioning the effects of the parent materials and their transformation products on soil microorganisms. We designed our investigations to definitively resolve the toxicity of individual nitroaromatic EMs and NG to the soil microbial activity endpoints and to critical processes in the soil ecosystem regulated by this community.

Our results showed that soil contamination with 2,4-DNT, 2-ADNT, 4-ADNT, and NG can alter the rates of biologically-mediated processes in soil by either inhibiting or stimulating the soil microbial activity at the affected sites. Ecotoxicological benchmark values determined in our studies were generally comparable to those reported for the effects of TNT, which was the most investigated nitroaromatic EM. However, the majority of ecotoxicological data for soil microorganisms determined in the TNT studies were derived from amended growth media or soil slurries; therefore, those data cannot be used directly to infer the exposure effects in aerobic upland soil, such as those used in our studies (USEPA, 2005), because the differences in the bioavailability and the fate of EMs in amended media can be substantial.

The SIR is directly related to the size of microbial biomass and the activity of soil microbial communities (Schmidt, 1992; Colores *et al*., 1996). The EC_{20} value of 878 mg/kg determined for 2,4-DNT in the present SIR study was within the 95% CI concentrations of the EC_{20} value of 530 mg/kg determined by Gong *et al*. (2000) in a SIR study using TNT-amended garden soil (8.7% OM, 29.5% clay, pH 6.5). The 28-d BR-based EC_{50} estimates determined in our studies for 2,4-DNT, 2-ADNT, 4-ADNT were an order of magnitude lower compared with either SIR-based estimates determined in our studies or in SIR studies with TNT reported by Gong *et al*. (2001). These EC_{50} estimates were consistent with the more sensitive indicator of carbon cycle impairment, such as the metabolic quotient, based on the EC_{50} and EC_{20} values of 35 and 3 mg/kg, respectively, established for TNT by Frische and Hoper, (2003).

The EC_{50} value of 1446 mg/kg determined in our SIR study for 2,4-DNT was similar to the EC_{50} value of 1122 mg/kg determined in our litter decomposition study with 2,4-DNT. This similarity is likely a result of correspondence between the microbial communities in SSL soil batches used in respective assays, and the pattern of responses of these microbial communities to addition of exogenous carbon sources (i.e., glucose and plant residue, respectively) to the soil. The EC_{50} value of 325 mg/kg determined for 2,4-DNT effect on microbial biomass C in the present study was similar to the EC_{50} of 362 mg/kg determined for TNT effects on actinomycetes, as assessed

by the prevalence of phospholipid fatty acids (PLFA) under *in situ* soil conditions of Joliet Army Ammunition Plant (Joliet, IL, USA; Fuller and Manning, 1998). An average EC_{50} value of 7.8 µg/mL was reported for TNT by Fuller and Manning (1997) for cell growth inhibition of 14 different Gram-positive bacterial isolates. Exposure to TNT was reported to lead to widespread changes in microbial community composition (Siciliano and Greer, 2000) and decreased diversity (Siciliano *et al*., 2000a). An increase in TNT concentration from 10 to 80 µg/mL resulted in lower diversity as measured by a decrease in the number of PLFAs from 34 to 14 (Fuller and Manning, 2004). Actinomycetes were reported to be more sensitive to TNT compared to Gram-positive organisms, but the precise reason why TNT is more toxic to actinomycetes is unclear at the present time (Fuller and Manning, 1998).

Inhibition of DH and PN activities by TNT was demonstrated by Gong *et al*. (1999a). The EC_{50} values for inhibition of activity ranging from 139 to 493 mg/kg for DH, and from 39 to 316 mg/kg for PN (Gong *et al*., 1999a), compare favorably with the results of our studies with nitrotoluenes; although the EC_{50} level of effect (50 mg/kg) could be estimated only for DH activity in the present study with 2,4-DNT. In our remaining studies that established a concentration-response relationship, inhibition of enzymatic activities could be reliably estimated only at the EC_{20} levels. Nitrification is the commonly used term that combines the activities of two distinctly different groups of microorganisms, ammonia oxidizing bacteria and nitrite oxidizing bacteria. In combination, these two groups of organisms convert ammonia to nitrate and derive energy from this process. Our studies showed that the effects of 4-ADNT on the PN activity were comparable with those established for TNT by Gong *et al*. (1999a; 2001) based on the reported EC_{50} values of 39 and 227 mg/kg.

Overall, the results of our studies and those reported in literature show that assessment of the soil microbial activity endpoints provides valuable information on the EM effects on critical ecosystem-level processes such as energy and nutrient cycling, and can complement and expand upon the ecotoxicological significance of data from the standardized single-species toxicity tests, thereby meeting DoD stewardship goals plus promoting management for sustainable use of military ranges.

8. Derivation of Soil Screening Concentrations

8.1. Derivation of Draft Ecological Soil Screening Levels

Soil contaminated with energetic materials may pose significant risks to military personnel, the surrounding environment, and offsite human and ecological receptors, thereby jeopardizing the long-term sustainability of military ranges and training sites. Although available data showed that some energetic materials can be persistent and highly mobile in the environment, their effects on terrestrial ecological receptors had not been sufficiently investigated. Therefore, development of ecotoxicological benchmarks for EM in soil has become a critical need in the United States.

Assessment and protection of the terrestrial environment at defense installations can be advanced by developing and applying scientifically based Ecological Soil Screening Levels (Eco-SSL; http://www.epa.gov/ecotox/ecossl/SOPs.htm, accessed 19October2012) for energetic materials released into upland aerobic soil environments (USEPA, 2005). Eco-SSL values are concentrations of contaminants in upland aerobic soil that when not exceeded are deemed protective of ecological receptors that commonly come into contact with soil or ingest biota that live in or on such soils. These values can be used in the SLERA to identify those contaminants that are not of potential ecological concern in soils, thus do not require further evaluation in the BERA, potentially resulting in cost-savings during ecologically-based site assessments and remedial investigations. Our extensive literature review (Kuperman *et al.*, 2009) showed that, despite considerable attention to assessing ecotoxicity of energetics, data previously available in literature for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG were insufficient to develop Eco-SSL values for soil invertebrates and terrestrial plants. Several definitive multi-year ecotoxicity studies were initiated, and conducted, to fill the existing data gaps (Kuperman *et al.*, 2003; 2006b; Rocheleau *et al.*, 2003; 2006; 2008; 2010; 2011; Simini *et al.*, 2003; 2006). These studies were designed to specifically meet the USEPA criteria (USEPA, 2005) for derivation of toxicity benchmarks acceptable for Eco-SSL development, and to expand ecotoxicological data that can aid the site managers in the knowledge-based decision making process for securing the sustainable use of testing and training installations. General concepts of USEPA Guideline (USEPA, 2005) or Eco-SSL development are summarized in this section of report in order to assist users in reviewing and interpreting its findings.

Selection of appropriate test methods and test species for toxicity testing to generate appropriate ecotoxicological benchmarks is among the important aspects in the process of developing benchmarks, and deriving a draft Eco-SSL. The USEPA preference for using standardized toxicity assays for generating benchmarks, and importance of ecological relevance of test species within the soil ecosystem were emphasized in USEPA guidance (USEPA, 2005). Several terrestrial toxicity tests have been developed, or improved by standardization, by different agencies and organizations. Leading among them are: the International Organization for Standardization (ISO), the American Society for Testing and Materials (ASTM), Environment Canada (EC), Organization for Economic Co-operation and Development (OECD), and USEPA. After an extensive review of existing standardized test methods, and based on the experience accumulated in the previous ecotoxicity studies, ASTM standard guide for conducting terrestrial plant toxicity tests (ASTM, 2002), and USEPA early seedling growth toxicity test (USEPA,

1996) were selected for assessing EM effects on terrestrial plants. Toxicity studies were conducted using a dicotyledonous symbiotic species alfalfa, the monocotyledonous species barnyard grass, and perennial ryegrass. These three plant species were identified as species sensitive to the EM compounds tested, and having performance parameters in SSL or TSL soil that are sufficient to meet the validity criteria required by the respective guidance for these standardized definitive toxicity tests.

Toxicity testing with soil invertebrates was conducted using the ISO assays for earthworms, potworms, and Collembola (springtails). The specific assays were: for earthworm, ISO/11268-2:1998 *Soil Quality – Effects of Pollutants on Earthworms* (*Eisenia fetida* Savigny 1826) – *Part 2: Determination of Effects on Reproduction* (ISO, 1998a); for potworm, ISO/16387 *Soil quality — Effects of pollutants on Enchytraeidae* (*Enchytraeus sp.*) *— Determination of effects on reproduction and survival* (ISO, 2004b), with the potworm species *E. crypticus* selected as the species for establishing benchmarks for draft Eco-SSL development; and for springtails, ISO/11267 *Soil quality —Inhibition of Reproduction of Collembola* (*Folsomia candida*) *by Soil Pollutants* (ISO, 1998b). Guidelines for these ISO assays were originally developed for use with OECD artificial soil (OECD, 1984). Similar artificial soil formulation was later adapted for USEPA Standard Artificial Soil (SAS; USEPA, 1996), and for ASTM artificial soil (ASTM E1676-04, 2004). However research studies, including those with EM compounds, have demonstrated that these ISO assays can be successfully adapted for use with natural soils (Amorim et al., 2009; 2005a,b; Dodard *et al*., 2005; Kuperman *et al*., 1999; 2003; 2004b; 2005; 2006a; 2006b; 2006d; Robidoux *et al*., 2002a; 2004c; Simini *et al*., 2003; 2006), necessary for establishing benchmarks for draft Eco-SSLs development.

Toxicological benchmarks utilized in the derivation of respective draft Eco-SSLs for each EM were determined in definitive studies on the basis of concentration-response relationships, using regression models selected from among those described in Environment Canada Guidance Document (EC, 2005). All benchmarks were established from analytical determinations using acetonitrile-extractable EM concentrations in soil, utilizing Method 8330A (USEPA, 2007). A draft Eco-SSL for an EM-receptor pairing (*e.g.*, NG-invertebrates) was calculated as the geometric mean of the EC_{20} toxicity benchmarks (*i.e.*, the 20 percent negative effect levels), determined in individual toxicity studies. Growth measurement endpoints, including fresh and dry shoot mass, were used for developing toxicity benchmarks for terrestrial plants. Reproduction measurement endpoints, including cocoon production and juvenile production for the earthworms, and juvenile production for the potworms and Collembola, were used for developing toxicity benchmarks for soil invertebrates. Selection of these measurement endpoints complied with USEPA requirement of using growth or reproduction endpoints for developing toxicity benchmarks in the derivation of Eco-SSLs for terrestrial plants and soil invertebrates, respectively (USEPA, 2005). The derivation process for these draft Eco-SSLs was completed separately for terrestrial plants and soil invertebrates, respectively, for each EM weathered-and-aged in soil. The minimum number of benchmarks required by USEPA to derive an Eco-SSL is three independent toxicity benchmark values, generated under specific conditions detailed within Eco-SSL Guidance (USEPA, 2005); research conditions met specified USEPA conditions and benchmarks exceeded that required by USEPA to derive an Eco-SSL.

Phytotoxicity benchmarks (EC_{20} values for growth inhibition endpoints) utilized for the derivation of the terrestrial plant-based draft Eco-SSL for 2,4-DNT weathered-and-aged in soil are presented in Table 66. These benchmarks were established in present studies with TSL soil

and separate studies with SSL soil reported by Rocheleau *et al*. (2010). A total of 12 phytotoxicity benchmarks developed in those studies were utilized to derive a draft Eco-SSL for 2,4-DNT, yielding an Eco-SSL value of 6 mg/kg (soil dry mass basis; Table 66).

Phytotoxicity benchmarks (EC_{20} values for growth inhibition endpoints) utilized for the derivation of the terrestrial plant-based draft Eco-SSL for 2-ADNT, 4-ADNT, and NG weathered-and-aged in soil are presented in Tables 67-69. These benchmarks were established in present studies with SSL soil. The nitramine HMX was not phytotoxic at ≥10000 mg/kg (nominal), the highest concentration tested in the limit test with the three plant species. Consequently, no draft Eco-SSLs for terrestrial plants could be developed for HMX.

Table 66. Derivation of Draft Eco-SSL values for 2,4-DNT weathered-and-aged in Sassafras sandy loam (SSL) or Teller sandy loam (TSL) soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), barnyard grass (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*).

Receptor Group	Soil*	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Alfalfa				
Fresh mass	SSL	7	2-11	
Dry mass	SSL	15	9-21	
Fresh mass	TSL	5	1-8	
Dry mass	TSL	7	0-15	
Barnyard grass				
Fresh mass	SSL	4	2-5	
Dry mass	SSL	6	5-8	6
Fresh mass	TSL	9	5-13	
Dry mass	TSL	9	2-15	
Ryegrass				
Fresh mass	SSL	5	4-7	
Dry mass	SSL	2	0-4	
Fresh mass	TSL	6	5-8	
Dry mass	TSL	8	6-11	

Table notes: *Toxicity data for 2,4-DNT established in our studies with SSL soil (corresponding soil batch designations is SSL2001) were reported previously in Rocheleau *et al.* (2010). All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Table 67. Derivation of Draft Eco-SSL values for 2-ADNT weathered-and-aged in Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), barnyard grass (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*).

Receptor Group	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Alfalfa			
Fresh mass	4	0-15	
Dry mass	36	0-134	
Barnyard grass			
Fresh mass	8	6-11	14
Dry mass	9	5-14	
Ryegrass			
Fresh mass	27	21-34	
Dry mass	22	17-27	

Table notes: All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Table 68. Derivation of Draft Eco-SSL values for 4-ADNT weathered-and-aged in Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), barnyard grass (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*).

Receptor Group	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Alfalfa			
Fresh mass	15	7-23	
Dry mass	11	0.1-23	
Barnyard grass			
Fresh mass	21	8-33	33
Dry mass	21	7-34	
Ryegrass			
Fresh mass	127	78-176	
Dry mass	130	73-188	

Table notes: All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Table 69. Derivation of Draft Eco-SSL values for NG weathered-and-aged in Sassafras sandy loam soil using growth benchmarks for terrestrial plants alfalfa (*Medicago sativa*), barnyard grass (*Echinochloa crusgalli*), and perennial ryegrass (*Lolium perenne*).

Receptor Group	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Alfalfa			
Fresh mass	5	0-13	
Dry mass	83	25-141	
Barnyard grass			
Fresh mass	16	0-38	21
Dry mass	12	1-23	
Ryegrass			
Fresh mass	42	9-75	
Dry mass	26	12-41	

Table notes: All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Soil invertebrate toxicity benchmarks (EC_{20} values for reproduction endpoints) utilized for the derivation of the soil invertebrate-based draft Eco-SSL for 2,4-DNT weathered-and-aged in soil are presented in Table 70. These benchmarks were established in the present studies with TSL soil, and separate studies with SSL soil (Kuperman, 2003; Kuperman *et al.*, 2006; Simini *et al.*, 2006). A total of 8 invertebrate benchmarks developed in those studies were utilized to derive a draft Eco-SSL for 2,4-DNT, yielding an Eco-SSL value of 18 mg/kg (soil dry mass basis; Table 70).

Soil invertebrate toxicity benchmarks (EC_{20} values for reproduction endpoints) utilized for the derivation of the soil invertebrate-based draft Eco-SSL for 2-ADNT, 4-ADNT, and NG weathered-and-aged in soil are presented in Tables 71-73. These benchmarks were established in present studies with SSL soil. A total of 4 soil invertebrate toxicity benchmarks developed in those studies were utilized to derive draft Eco-SSL for each of the EMs, yielding the Eco-SSL values of 43, 18, and 13 mg/kg, respectively for 2-ADNT, 4-ADNT, and NG (Tables 71-73).

Soil invertebrate toxicity benchmarks (EC_{20} values for reproduction endpoints) utilized for the derivation of the soil invertebrate-based draft Eco-SSL for HMX weathered-and-aged in soil are presented in Table 74. These benchmarks were established in present studies with TSL soil and separate studies with SSL soil reported by Kuperman *et al*. (2003) and Simini *et al*. (2003). A total of 3 soil invertebrate benchmarks developed in those studies were utilized to derive a draft Eco-SSL for HMX, yielding an Eco-SSL value of 16 mg/kg (soil dry mass basis; Table 74).

Table 70. Derivation of Draft Eco-SSL values for 2,4-DNT weathered-and-aged in Sassafras sandy loam (SSL) or Teller sandy loam (TSL) soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*.

Receptor Group	Soil[†]	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm				
Cocoon production	SSL	25	16-35	
Juvenile production	SSL	29	17-42	
Cocoon production	TSL	20	8-32	
Juvenile production	TSL	5	0-11	
Potworm				18
Juvenile production	SSL	14	10-18	
Juvenile production	TSL	28	21-34	
Collembola				
Juvenile production	SSL	15	11-19	
Juvenile production	TSL	24	20-28	

Table notes: [†]Toxicity data for 2,4-DNT established in our previous studies with SSL soil (Kuperman, 2003; Kuperman *et al.*, 2006; Simini *et al.*, 2006) are included in this table. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Table 71. Derivation of Draft Eco-SSL values for 2-ADNT weathered-and-aged in Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*.

Receptor Group	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm			
Cocoon production	48	40-56	
Juvenile production	31	13-49	
Potworm			43
Juvenile production	76	63-88	
Collembola			
Juvenile production	30	26-34	

Table note: All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Table 72. Derivation of Draft Eco-SSL values for 4-ADNT weathered-and-aged in Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*.

Receptor Group	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm			
Cocoon production	17	12-22	
Juvenile production	12	7-18	
Potworm			18
Juvenile production	21	9-32	
Collembola			
Juvenile production	26	19-33	

Table note: All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Table 73. Derivation of Draft Eco-SSL values for NG weathered-and-aged in Sassafras sandy loam soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*.

Receptor Group	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm			
Cocoon production	24	14-34	
Juvenile production	21	11-31	
Potworm			13
Juvenile production	44	11-77	
Collembola			
Juvenile production	1.3	0.5-2.1	

Table note: All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Table 74. Derivation of Draft Eco-SSL values for HMX weathered-and-aged in Sassafras sandy loam (SSL) or Teller sandy loam (TSL) soil using reproduction benchmarks for earthworm *Eisenia fetida*, potworm *Enchytraeus crypticus* and collembolan *Folsomia candida*.

Receptor Group	Soil[#]	EC_{20} (mg/kg)	95% Confidence Internals (mg/kg)	Draft Eco-SSL (mg/kg)
Earthworm				
Cocoon production	SSL	>562[†]	ND	
Juvenile production	SSL	>562[†]	ND	
Cocoon production	TSL	2	0.6-3.3	
Juvenile production	TSL	2	0.5-4.4	
Potworm				16
Juvenile production	SSL	>17500[††]	ND	
Juvenile production	TSL	>10208[‡]	ND	
Collembola				
Juvenile production	SSL	1046	58-2000	
Juvenile production	TSL	>10208[‡]	ND	

Table notes: [#]Toxicity data for HMX weathered-and-aged in SSL soil were established in SERDP CU-1221 project (Kuperman, 2003). ND=Not determined; [†]Simini *et al.*, 2003 (value was not used for derivation of Eco-SSL); [††]Kuperman *et al.*, 2003 (value was not used for derivation of Eco-SSL); [‡]Value was not used for derivation of Eco-SSL. All values are based on acetonitrile extractable concentrations (USEPA Method 8330A).

Review of ecotoxicological benchmarks utilized in deriving draft Eco-SSLs shows that, although majority of values were fairly uniform, there were instances of substantial variability among the EC_{20} estimates determined in toxicity tests. In phytotoxicity testing, the greatest difference was found for 4-ADNT effects on alfalfa and ryegrass growth (dry mass) benchmarks, which ranged from 11 mg/kg for alfalfa to 130 mg/kg for ryegrass. In soil invertebrate toxicity testing, greatest differences were found for HMX effects on juvenile production benchmarks, which ranged from 2 mg/kg for earthworm to 1046 mg/kg for Collembola, while reproduction of potworms was not affected by HMX ≤17500 mg/kg in SSL soil, and ≤10208 mg/kg in TSL soil. These examples of species-specific variability in respective toxicities provide clear evidence in support of the USEPA requirement for use of multiple species for generating ecotoxicological benchmarks for Eco-SSL development, and for having selection criteria for determining which data are most appropriate for developing Eco-SSL values.

The draft Eco-SSLs are intentionally conservative, but scientifically realistic, in order to provide confidence that contaminants that potentially present an unacceptable risk are not screened out early in the SLERA process. This conservative nature of Eco-SSLs developed in this report for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG was achieved by: 1) using natural soils that have properties that support high relative bioavailability of these EM to ecologically relevant test species, 2) using growth (for terrestrial plants) or reproduction (for soil invertebrates)

measurement endpoints for benchmark derivation, 3) relying on a low effect level (EC_{20}; 20 percent reduction in comparison to carrier control) on respective measurement endpoints, 4) using the geometric mean of the respective benchmarks to establish an Eco-SSL value (*i.e.*, more conservative than an arithmetic mean). By deriving conservative soil screening values protective of these receptor groups, when these respective 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG Eco-SSL values for soil invertebrates and plants are used in conjunction with corresponding values developed for avian wildlife and mammalian wildlife, it is assumed that the terrestrial ecosystem will be protected from unacceptable adverse effects associated with upland aerobic soil that is contaminated with 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG.

These draft Eco-SSLs are applicable to all sites where key soil parameters fall within a certain range of chemical and physical parameters (USEPA, 2005). They apply to upland aerobic soils where: the pH is greater than or equal to 4.0 and less than or equal to 8.5 and the organic matter content is less than or equal to 10%. The majority of soil toxicity tests that were reported in literature utilized SAS with high organic matter content (10%), which limited their usefulness for Eco-SSL derivation. In contrast, ecotoxicological benchmarks utilized in this report for developing draft Eco-SSLs for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG were established in toxicity studies using natural soils that met the criteria for Eco-SSL development, in large part, because they are aerobic upland soils that have characteristics supporting high relative bioavailability of EMs (low organic matter and clay content; Table 1). This was necessary to ensure that draft Eco-SSLs for terrestrial plants and soil invertebrates developed in this project are adequately conservative for a broad range of soils within the specified boundary conditions (USEPA, 2005).

Derivation of Eco-SSL values prioritizes ecotoxicological benchmarks that are based on measured soil concentrations of a chemical over those based on nominal concentrations (USEPA, 2005). The exposure concentrations of each EM in soil were analytically determined in all definitive tests from which benchmarks were determined, reported, and utilized in the derivation of the draft Eco-SSL values included in this report. Analytical determinations were performed utilizing the USEPA Method 8330A (USEPA, 2007), both for extraction of EMs from soil and for measuring acetonitrile-extractable chemical concentrations. Furthermore, special consideration was given to the inclusion of weathering-and-aging of contaminant explosives in soil in the assessment of the EM effects on terrestrial receptors, for use in draft Eco-SSL development. Consequently, ecotoxicological benchmarks for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG, each independently weathered-and-aged in TSL or SSL soil, more closely approximate the exposure conditions in the field, compared to benchmarks established in studies with freshly amended soil; these are more relevant for ERA because Eco-SSL development by USEPA was specifically undertaken for use at Superfund sites, which are locations where contaminants have been long-present (a situation similar to DoD testing and training ranges).

Inclusion of species from different taxonomic groups, representing a range of sensitivities, was an important consideration for selecting the test battery for Eco-SSL development because the respective sensitivities often correlate with physiologically-determined modes of toxic action, and can vary among taxa. The selected species were expected to represent the spectrum of diverse ecological functions that are attributed to organisms comprising soil communities: primary producers, and different functional groups of soil invertebrates. Test species selected for

the studies are representative surrogates of species that normally inhabit a wide range of site soils and geographical areas (*i.e.*, the species are ecologically relevant). The exposure focused upon for terrestrial plants is direct contact with EMs in soils, and for soil invertebrates the exposures focused upon ingestion of EM-contaminated soil as well as direct contact exposures (USEPA, 2005). These exposures were considered under conditions of high relative bioavailability of EM in SSL or TSL soil. The terrestrial plant and soil invertebrate species tested are sensitive to a wide range of contaminants, and represent different routes of exposure (e.g., ingestion, and dermal absorption within soil for soil invertebrates, and uptake from soil solution for plants). Finally, selected terrestrial toxicity tests with representative test species, have been standardized and generate reproducible, statistically-valid results, which imparts a greater confidence in the data, and generates less uncertainty associated with the decisions and recommendations that are based on the test data, both of which are important factors for draft Eco-SSL development.

In order to ensure that draft Eco-SSL values developed in this project comply with all criteria, and would obtain the highest score in each selection criteria category, experimental designs of toxicity tests used to establish the respective benchmarks for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG were evaluated using the Literature Evaluation Criteria accepted by the Eco-SSL Workgroup (USEPA, 2005), summarized in Table 75. Such review should expedite USEPA concurrence with the results of these investigations, and acceptance of the derivations of the respective 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG draft Eco-SSL values.

Table 75. Summary of Literature Evaluation Process for Plant and Soil Invertebrate Eco-SSLs (modified from USEPA, 2005).

Criteria	Rationale
1: Testing was Done Under Conditions of High Bioavailability.	Bioavailability of metals and polar organic compounds is influenced by pH and soil organic matter, cationic exchange capacity, and clay content. The scoring is intended to favor relatively high bioavailability.
2A: (Laboratory) and **2B:** (field): Experimental Designs for Studies are Documented and Appropriate.	Experimental design can significantly influence the quality of a study. Higher quality studies will use an experimental design sufficiently robust to allow analysis of the test variables and discriminate non-treatment effects.
3: Concentration of Test Substance in Soil is Reported.	The concentration of the contaminant tested must be reported unambiguously.
4: Control Responses are Acceptable**.**	Negative controls are critical to distinguish treatment effects from non-treatment effects.
5: Chronic or Life Cycle Test was Used**.**	Chronic toxicity tests assessing long-term adverse sub-lethal impacts on the life-cycle phases of an organism are considered superior to acute toxicity tests.
6: Contaminant Dosing Procedure is Reported and Appropriate for Contaminant and Test.	Contaminant dosing procedure may affect the outcome of a test. Dosing procedure should include: (A) The form of the contaminant; (B) The carrier or vehicle (e.g., solvent, water, etc.); (C) How the carrier was dealt with following dosing (i.e., allowed to volatilize, controls, etc.); (D) procedure for mixing of soil with contaminant (homogeneity).
7: A Dose-Response Relationship is Reported or can be Established from Reported Data.	Two methodologies that can be used to identify this benchmark concentration. The first method generates a no observed effect concentration (NOEC) and a lowest observed effect concentration (LOEC). The second method uses a statistical model to calculate a dose response curve and estimate an effect concentration for some percentage of the population (EC_x), usually between an EC_5 and an EC_{50}.
8: The Statistical Tests used to Calculate the Benchmark and the Levels of Significance were Described.	Statistical tests and results reported in the study should be sufficient to determine the significance of the results.
9: The Origin of the Test Organisms is Described.	The results of a toxicity test can be influenced by the condition of the test organisms. Culture conditions should be maintained such that the organisms are healthy and have had no exposure above background to contamination prior to testing (invertebrates) or detailed information is provided about the seed stock (plants).

Information relevant for each criterion of the evaluation processes is summarized below.

1. Natural soils, Teller sandy loam (Fine-loamy, mixed, active, thermic Udic Argiustoll) or Sassafras sandy loam (Fine-loamy, siliceous, mesic Typic Hapludult) were utilized in the studies to assess the EM toxicity for the chosen test species. These soils were selected for

developing ecotoxicological values protective of soil biota because they are upland aerobic soils that have physical and chemical characteristics supporting high relative bioavailability of the test chemicals (USEPA, 2005).

2. Toxicity assays were conducted to determine the effects of 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG on terrestrial plants and soil invertebrates. Testing was designed to specifically meet the criteria required for Eco-SSL development. All methods used are documented within the cited publications, and include detailed accounts of individual studies. All assays included both range-finding tests to bracket 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG concentration range for each test species, and definitive tests to determine ecotoxicological benchmarks required for development of draft Eco-SSL values.

3. Nominal concentrations were analytically verified in all definitive test treatments. All ecotoxicological parameters were estimated using measured chemical concentrations for each treatment level.

4. Each toxicity test was appropriately replicated, and included negative (no chemicals added), positive (reference chemical), and carrier (acetone) controls. Test validity criteria were used in all definitive assays. Validity criteria in definitive toxicity tests with terrestrial plants specified minimal percent germination in negative controls for each species tested, and the quality control limit for EC_{50} values in positive control (boric acid). Validity criteria for negative controls in the definitive toxicity tests with soil invertebrates specified minimal percent adult survival, minimal number of juveniles produced, boundaries for coefficient of variation for reproduction. The EC_{50} values determined in positive control (boric acid) for reproduction measurement endpoint were monitored throughout the project using Warning Charts.

5. Toxicity tests were based on the assessments of EM effects on growth (for plants) and reproduction (for soil invertebrates); although not utilized in the derivation of Eco-SSL values, the additional endpoints seedling emergence and adult survival, respectively, were determined for comparison to the historic database of acute measurement endpoints.

6. Soil amendment procedures were documented, and these included the form of EMs used, analytical purity of each EM, procedures for preparation of treatment concentrations using acetone carrier, time allowed to volatilize acetone in chemical hood, and duration of three-dimensional mixing to ensure the homogeneity of EM incorporation in test soil.

7. Toxicity data were analyzed using appropriate regression models to establish concentration-response relationships for each EM-test species measurement endpoint pairing. The EC_{20} (and EC_{50} values) for seedling emergence and growth endpoints in the phytotoxicity assays, and for cocoon/juvenile production in the soil invertebrate assays, were determined using SYSTAT software. The EC_{20} benchmark is preferred for deriving Eco-SSL values. The EC_{50}, a commonly reported benchmark, was included to enable comparisons of the results produced in this study with results reported by other researchers.

8. Statistical tests included regression analyses, and Analysis of Variance (ANOVA). Regression analyses were performed using SYSTAT software, version 11 (Systat Inc., Chicago, IL). Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Variances of the residuals were examined to decide whether or not to weight the data, and to select appropriate mathematical models. The asymptotic standard error (a.s.e.) and 95% confidence intervals (CI) associated with the point estimates were determined. Concentration-response relationships, mathematically modeled, are preferred for establishing benchmarks for use in Eco-SSL derivation (USEPA, 2005), and these were utilized to derive the draft Eco-SSL values in this report. ANOVA was used to determine the bounded No Observed Effect Concentration (NOEC) and Lowest Observed Effect Concentration (LOEC) values. Mean separations were done using Fisher's-Least-Significant-Difference (FLSD) pairwise comparison tests. A significance level of $p \leq 0.05$ was accepted for determining the NOEC and LOEC values. Student's *t*-Test (two-tailed) with significance level set at $p \leq 0.05$ was used in the limit tests with plants and invertebrates exposed to HMX using EXCEL 2007 software (Microsoft Corporation).

9. Sources of seed stocks and soil invertebrates included:
    - Alfalfa (variety Canada no. 1; Cat. # 550, Lot packed and tested 2000). Supplier: Williams Dam Seeds Ltd., Box 8400, Dundas Ontario, Canada, L9H 6M1.
    - Nitrogen-fixing bacteria for alfalfa (Nitragin Gold; Cat. # 309-9, Lot #NGA33). Supplier: Labon Inc. 1350 Newton, Boucherville, Quebec, Canada, J4B 5H2.
    - Barnyard grass/Japanese millet (variety Common no. 1; Cat. # 300-380, Lot # 9-6). Supplier: Labon Inc. 1350 Newton, Boucherville, Quebec, Canada, J4B 5H2.
    - Perennial ryegrass (variety Express; Cat. # 1269). Supplier: Pickseed Canada Inc., St-Hyacinthe, Quebec, Canada.
    - All soil invertebrate test species used in toxicity assays came from cultures maintained by the Environmental Toxicology laboratory, U.S. Army Edgewood Chemical Biological Center, APG, MD, USA.
    - Bioaccumulation tests were performed at the Biotechnology Research Institute (BRI), National Research Council Canada, Montreal, Quebec, Canada. Earthworm *Eisenia andrei* cultures were maintained at BRI and were purchased from Carolina Biological Supply Company, 2700 York Road, Burlington, NC, USA, 27215-3398.

A review of the information provided (above) for each criterion shows that experimental design of ecotoxicological investigations complied with all screening criteria used by the Eco-SSL Workgroup during literature evaluation processes for selecting or developing terrestrial plant and soil invertebrate benchmarks for deriving Eco-SSL values.

Benchmark data and draft Eco-SSL values developed in these studies have been transitioned to the USEPA (Eco-SSL Workgroup POCs Drs. David Charters and Marc Greenberg, USEPA Environmental Response Team), which is having the rules of selection applied to our results in order to determine the most appropriate benchmarks, and establish respective Eco-SSL values endorsed by USEPA for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG. Our draft Eco-SSL values, along with corresponding information, have also been transitioned to the Tri-Service

Environmental Risk Assessment Work Group (TSERAWG) in invited presentations to participating personnel representing Army, Navy, Air Force, USEPA, and their respective contractors involved in ERA. Thus, these values are available for use in ongoing ERA at military sites. Following endorsement by the USEPA, these Eco-SSL values will be made officially available for use in ERA of terrestrial habitats at military testing and training sites.

8.2. Derivation of Biological Activity-based Soil Screening Concentrations

Assessment of soil microbial activity can provide valuable information on the effects of energetic materials on critical ecosystem-level processes such as energy and nutrient cycling. Multiple soil microbial activity endpoints can be used to develop Biological Activity-based Soil Screening Concentrations (BA-SSC), utilizing an approach similar to Eco-SSL derivation described in section 4.5. The results of present studies, described in sections 4.4.1, 4.4.2, and 4.4.3, showed that soil contamination with 2,4-DNT, 2-ADNT, 4-ADNT, and NG can alter the rates of biologically-mediated processes in soil by either inhibiting or stimulating soil microbial activity. These results also demonstrated clearly that toxicity benchmarks for EM and the corresponding BA-SSC values can be reliably established for soil biological processes, and comply with stipulations and requirements developed for Eco-SSL values. Toxicity benchmarks (EC_{20} level or MATC) for EM effects on BR, SIR, microbial biomass C, litter decomposition (orchard grass), and enzyme activities in SSL soil presented in Table 76 in **bold font** were used for derivation of BA-SSC values. These BA-SSC values were derived using the same approach described in section 4.5 of this report for Eco-SSL development.

Table 76. Toxicity benchmarks used for development of Biological Activity-based Soil Screening Concentrations.

Microbial endpoints	2,4-DNT mg/kg	2-ADNT mg/kg	4-ADNT mg/kg	NG mg/kg
BR	**19**	**17**	**9**	**163**
SIR	**878**	>6078	>7819	**25**
Biomass C	**105**	**211**[†]	**1663**	**24**
Litter decomposition	**361**	>10000	>12560	**277**
AP	ND	**830**	**90**	**2180**[†]
DH	**16**	**415**	**28**	**15**
NAG	**122**	**375**[†]	>12560	ND
PN	ND	**175**	**113**	ND

Table notes: Values are soil concentration determined by USEPA Method 8330A. BR=basal respiration; SIR=substrate-induced respiration; PN=potential nitrification; DH=dehydrogenase; AP=acid phosphatase; NAG=N-acetyl-glucosaminidase; [†]MATC=Maximum Allowable Toxic Concentration (geometric mean of the NOEC and LOEC values); ND=Not Determined (no concentration-dependent effect within the concentration range tested).

The BA-SSC values shown in Table 77 can be recommended for use in Screening-Level Ecological Risk Assessment (SLERA) of aerobic EM-contaminated soils when microbial activity is included in the assessments. Data in Table 77 show that either terrestrial plant-based or soil invertebrate-based Eco-SSL values were more conservative (lower) than BA-SSC values in SSL soil, thus suggesting that biologically-mediated processes will be adequately protected in aerobic sandy loam soils contaminated with 2,4-DNT, 2-ADNT, 4-ADNT, or NG, when SLERA includes these two traditional receptor groups. Assessment of the soil microbial activity endpoints can complement and expand upon ecotoxicological significance of data from standardized single-species toxicity tests.

Table 77. Biological Activity–based Soil Screening Concentrations (BA-SSC) and draft Eco-SSL values for 2,4-DNT, 2-ADNT, 4-ADNT, and NG.

Energetic Material	Biological Activity BA-SSC (mg/kg)	Terrestrial Plant Eco-SSL (mg/kg)	Soil Invertebrate Eco-SSL (mg/kg)
2,4-DNT	104	6	18
2-ADNT	208	14	43
4-ADNT	84	33	18
NG	98	21	13

Table notes: Values are soil concentration determined by USEPA Method 8330A. Derivation of Eco-SSL values for terrestrial plants and soil invertebrates is shown in Tables 64-72 of this report.

9. Bioaccumulation studies

Bioaccumulation is the accumulation or uptake of a chemical in biological tissues (usually measured as a tissue concentration – mg chemical per kg tissue; NRC, 2003), which includes direct and indirect accumulation processes. Bioaccumulation in animals involves a number of interacting physiological processes that govern the uptake of a contaminant by an organism following dermal absorption of the contaminant, as well as its ingestion and subsequent absorption via the gut. These concepts have been described by Vijver *et al.* (2005). The accumulation of a chemical from the soil to an organism can be measured under steady state conditions as the ratio of tissue-to-soil concentrations (Van Gestel and Ma, 1988; Ma *et al*., 1995), and is referred to in this report as the bioaccumulation factor (BAF). Bioconcentration is the ratio of a chemical taken by an organism (e.g. fish) and the amount in water (BCF).

In addition, bioaccumulation can be calculated from the ratio of the uptake and the elimination kinetic rate constants and is termed kinetic bioaccumulation factor (BAF_K). This method involves the determination of individual rate constants for a test chemical during the uptake and elimination phases of a bioaccumulation test (Jager *et al*., 2005; Vijver *et al*., 2005).

We designed our studies to test the hypotheses that selected explosives released in soil can accumulate in soil invertebrates or terrestrial plants. We tested the hypothesis that EM can accumulate in the earthworm *E. andrei* by establishing the BAF values from tissue-to-soil concentration ratios determined in studies with non-labeled EM, and by establishing the BAF_K values from the uptake and elimination rate constants determined in studies with ^{14}C-EM. Values greater than one for calculated BAF or BAF_K were accepted as indications of EM accumulation in the earthworms under soil-tissue equilibrium or steady state conditions. Additional studies were conducted with RDX, and permit to test the effects of RDX concentration in soil, soil hydration rate, earthworm loading rate, and duration of exposure on RDX accumulation by the earthworms. Uptake of EM by plants was assessed using either non-labeled or ^{14}C-EM to determine the BCF in studies with ryegrass *L. perenne*. The mass balance studies were conducted with the ^{14}C-EM using Sassafras sandy loam soil and the plant microcosm system.

In this report the term, BAF is used for earthworms and bioconcentration (BCF) is used for plant results and is the ratio of the chemical found in earthworm or plant tissue to its amount in soil for a certain exposure period. The nomenclature BCF is used for plant assuming that any chemical found in plants should result from the movement of chemical via water adsorption from soil.

9.1. Bioaccumulation of RDX

9.1.1. Effect of RDX in soil on the accumulation of RDX by earthworms using both the empirical BAF and kinetic models

The manuscript Sarrazin *et al.* (2009) has been published and is attached in appendix.

9.1.2. Role of interstitial water in the accumulation of RDX by earthworms

The manuscript Savard *et al.* (2010) has been published and is attached in appendix.

9.1.3. Effect of the soil hydration level on the uptake of RDX by earthworms

Nominal concentrations of RDX in TSL soil were confirmed by chemical analysis of acetonitrile extracts (USEPA Method 8330A, 2007) from bulk soil (Table 78). Fractions of the "total" RDX recovered from TSL soil was extracted from interstitial water using the method described in the 2006 ER-1416 Annual Report. The concentration of RDX in interstitial water in TSL soil is reported in Table 78. Similar values were obtained for either 75 or 95% soil hydration levels. A product of RDX degradation, MNX, was detected only in the interstitial water of soil samples hydrated to 95% of the WHC (Table 78).

Table 78. Concentrations of RDX in Teller sandy loam soil hydrated to 75 or 95% of the water holding capacity (WHC).

Extraction medium	RDX Measured in 75% WHC	RDX Measured in 95% WHC	MNX Measured in 75% WHC	MNX Measured in 95% WHC
Bulk soil (mg/kg)	10.40 ± 0.89	10.62 ± 1.09	ND	ND
Interstitial water (mg/L)	15.76 ± 2.04	15.56 ± 0.27	ND	0.32 ± 0.28

Table notes: Values are means ± standard errors (n=3). ND = not detected (detection limit = 0.15 mg/kg or 0.03 mg/L).

Data indicated that the tissue concentration of RDX in the earthworms reached a plateau after 2 d of exposure (Figure 67). Analyses suggest that accumulation of RDX in earthworms in TSL hydrated to either level tested could be described by the exponential equation:

$$\mathbf{Y = Y_{max} \times (1 - e^{-kt})}$$

where Y is the concentration of RDX in tissue (mg/kg), Y_{max} is the maximal concentration of RDX found in the earthworm tissue (mg/kg) at a steady-state, k is the uptake rate constant (d^{-1}), and t is the duration of uptake (in days). Results of regression analyses showed that values for Y_{max} and k were independent of the soil hydration level based on the 95% confidence intervals (Table 79).

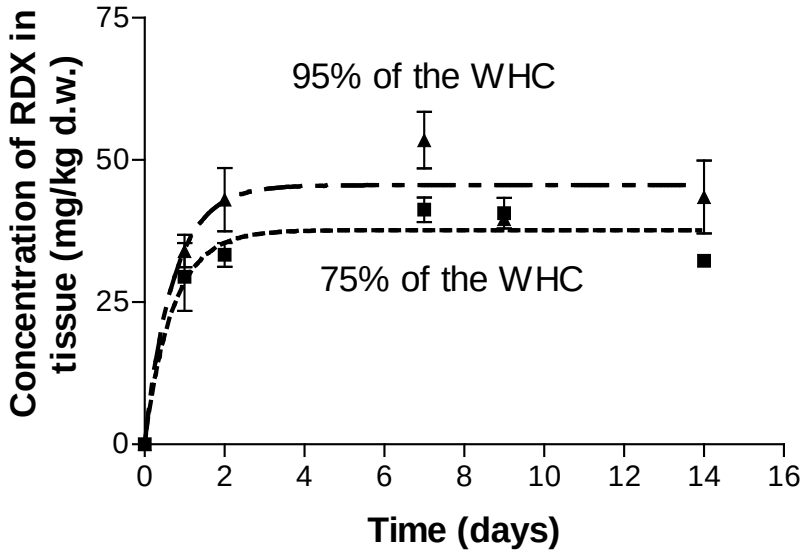


Figure 67. Uptake of RDX by *Eisenia andrei* during a time-series exposure in Teller sandy loam soil amended with 10 mg RDX/kg and hydrated to 75 or 95% of the water holding capacity.
Results are expressed as means and standard errors (n=3). The squares (■) and triangles (▲) represent 75 and 95% of the water holding capacity, respectively.

Table 79. Regression analysis parameters for RDX uptake in *Eisenia andrei* exposed for 14 days in Teller sandy loam soil amended with 10 mg RDX/kg and hydrated to 75 or 95% of the water holding capacity (WHC).

Regression parameter	75% of the WHC	95% of the WHC
Maximum RDX concentration in tissue, Y_{max} (mg/kg)	37. 7	45.6
95% confidence intervals	33.9 – 41.4	40.4 – 50.8
Uptake rate constant, k (d^{-1})	1.4	1.4
95% confidence intervals	0.6 -2.2	0.5 – 2.3
Coefficient of determination, R^2	0.87	0.84

9.1.4. Effect of exposure to RDX in Teller sandy loam soil on the earthworm lipid content

The lipid content was analyzed in earthworms exposed up to 14 d in TSL soil freshly amended with 10 mg/kg RDX, or control soil (no RDX added). Results indicated a decrease in lipid content of earthworm following exposure in TSL soil. Lipid in earthworms exposed for 7d in TSL control soil and RDX amended soil were significantly lower than those from culture time 0 ($p=0.038$ and $7.7x10^{-5}$ respectively). The 30% decrease in the lipid content of earthworms

exposed in RDX amended soil versus control soil observed after 7d in TSL, was not significant (*p=0.198*) (Table 80). No relationship between the lipid content and RDX accumulation in earthworms could be established in this study.

Table 80. Lipid concentration in *Eisenia andrei* determined in a time series exposure to RDX-amended Teller sandy loam soil.

	Lipid content in earthworms (g/100 g wet weight)	
Exposure period (days)	Control soil	RDX-amended soil
0	4.1 ± 0.3 (n= 24)	
7	2.6 ± 0.6 (n= 6)	1.9 ± 0.4 (n=13)
14	2.1 (n=1)	1.5 ± 0.6 (n=5)

Table notes: Values are means ± standard errors; variable replication is shown in brackets.

9.1.5. Effect of the earthworm loading rate on ^{14}C-RDX uptake by the earthworms

Range-finding data indicated that the accumulation of ^{14}C-RDX in earthworms was affected by and directly proportional to the earthworm loading rate (Figure 68). The uptake (dpm/kg dry tissue) ranged from 67 to 135 in the one-earthworm-per-jar test, from 140 to 180 in the six-earthworms-per-jar test, and from 203 to 240 in the ten-earthworms-per-jar test. Uptake data was most variable when only one earthworm was added per test jar, compared to the other earthworm loading rates.

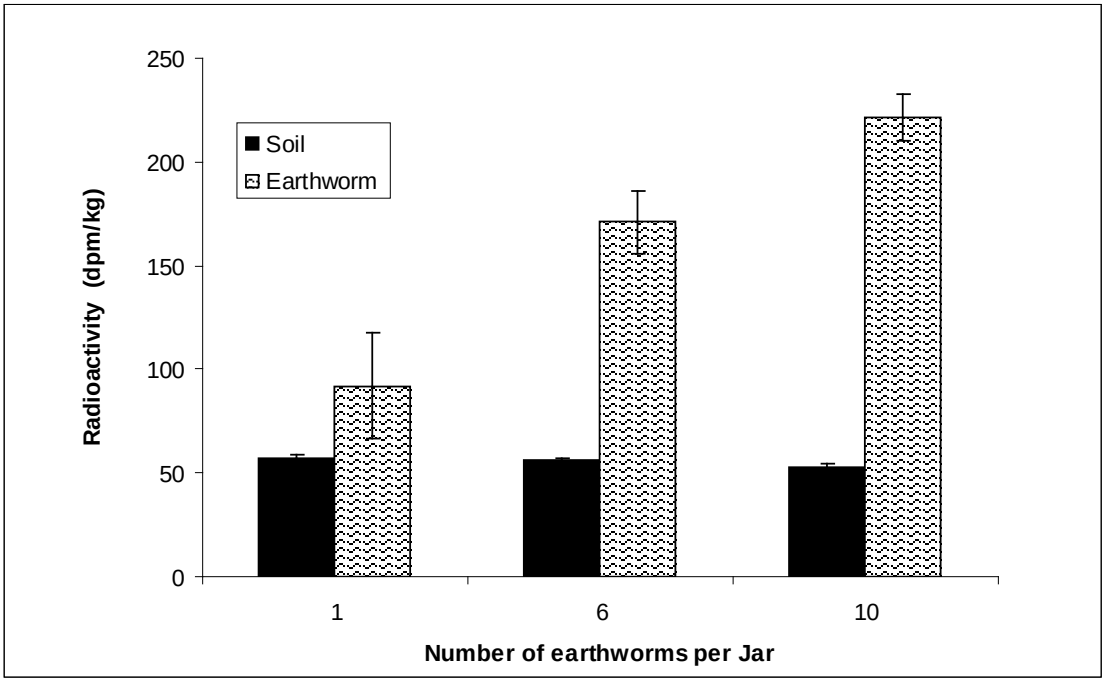


Figure 68. ^{14}C-activity in soil and earthworms following a 7-d exposure to 100 mg ^{14}C-RDX/kg Sassafras sandy loam soil.
Results are expressed as means and standard deviations (n=3).

9.1.6. Kinetic accumulation of ^{14}C-RDX in earthworms at low concentration

These experiments were performed to verify if the use of a lower concentration of EMs will increase the accumulation potential of the earthworms. Results showed that in accord with non-labeled studies, the BAF_k increased with decreasing concentration of RDX. Figure 69 shows the uptake and elimination of RDX exposed to 10 mg/kg ^{14}C -RDX. In these experiments, the maximum RDX in earthworms was $26.91 \times 10^{+3}$ dpm / g dry tissue (Table 81). This value corresponds to an equivalent amount of 40 µg RDX/g dry tissue, and was consistent with published results for 10ppm exposed RDX (Savard *et al*. 2010). The equations described in section 9.1.3 were used to calculate a constant kinetic of uptake (k1) of 9.1 g soil/g tissue/d, and a a constant kinetic of elimination (k2) of 1.2 d^{-1}. The calculated BAF_k for earthworm exposed to 10 ppm RDX in SSL soil was 7.35 g soil/g tissue and was consistent with results (6.0) published in Sarrazin *et al*. (2009) for earthworm exposed to 10 mg/kg non-labeled RDX in soil.

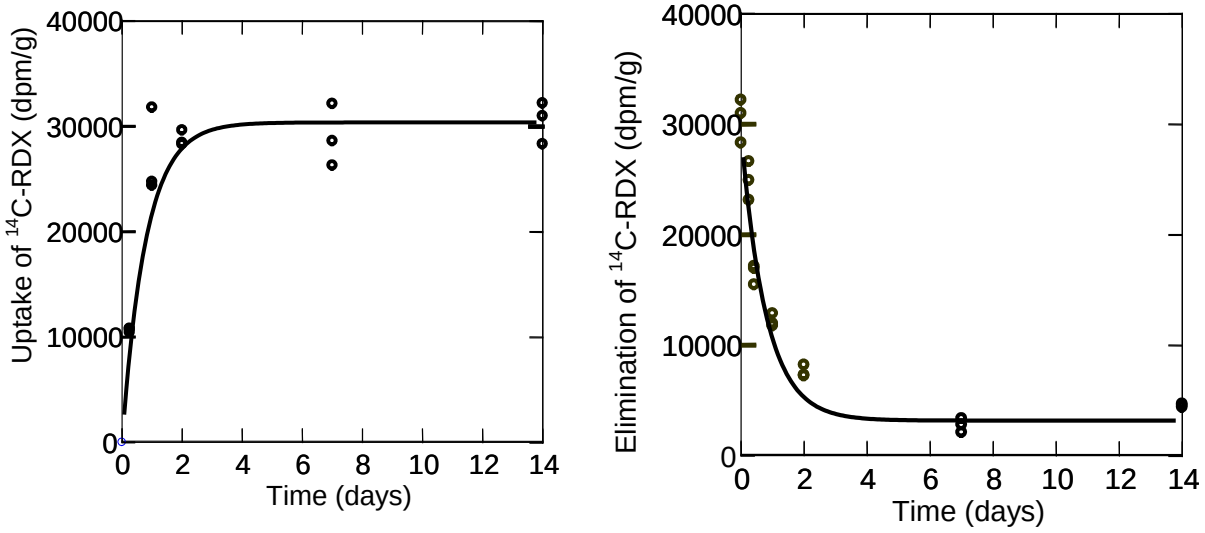


Figure 69. Kinetic of the accumulation and elimination of ^{14}C-RDX by earthworms following exposure in soil.

Table 81. The parameters of uptake and elimination for ^{14}C-hexahydro-1,3,5-trinitro-1,3,5-triazine (^{14}C-RDX) in the earthworm (*Eisenia andrei*)

Body residue [a] (x 10^3 dpm/g tissue)		Rate constants [a]		Coefficient of determination (R^2)		
$[RDX_T]_{SS}$ [b]	$[RDX_T]_R$ [b]	(k_1) (g soil/g tissue/d)	(k_2) (per day)	Uptake	Elimination	BAF_K [b]
26.99 (0.89)	3.56 (0.56)	9.05 (0.24)	1.23 (0.12)	0.96	0.98	7.35

Table notes: [a] Data are expressed as model estimate with the asymptotic standard error indicated in parentheses. Equations used for modeling were described in section 3.13.5. [b] $[RDX_T]_{SS}$ is the sum of the radioactivity in the acetonitrile extractable fraction of the earthworm (dpm/g dry wt tissue) under apparent steady-state conditions, $[RDX_T]_R$ is the residual radioactivity remaining in the extractable fraction of the earthworm (dpm/g dry wt tissue) at the end of the experiment. Rate constants were determined using the model specified in the text; k_1 is the total ^{14}C-activity uptake rate constant (g dry wt soil/g dry wt tissue/d), and k_2 is the elimination rate constant (per day) for extractable ^{14}C-activity. The BAF_K is the kinetic-based bioaccumulation factor defined as k_1/k_2.

9.1.7. Uptake of non-labeled RDX in plants

9.1.7.1. Evaluation of the plant accumulation microcosm (PAM) for the plant uptake studies

Preliminary studies were done to determine whether the plant accumulation microcosm (PAM) could be used to measure RDX accumulation in plants. Figure 70 shows the accumulation of RDX in ryegrass shoots or roots following 21 or 34 d of exposure to RDX in SSL soil. These studies were done using test units placed in polyethylene bags or in the PAM. Data indicates that RDX accumulation in plant shoots was greater in the PAM (2648 and 4822 mg/kg after 21 and 34 d, respectively) compared with that in the bags (2216 and 1728 mg/kg after 21 and 34 d, respectively). Similar results were found for the roots (Figure 70). In addition, MNX (up to 20.5 mg/kg) was found in shoot samples. These data provided basis for selecting the PAM for the ^{14}C-RDX accumulation studies. Preferential accumulation of RDX in the ryegrass shoots compared with accumulation in the roots established in these preliminary studies agrees with the results of earlier studies by Rocheleau *et al*. (2008).

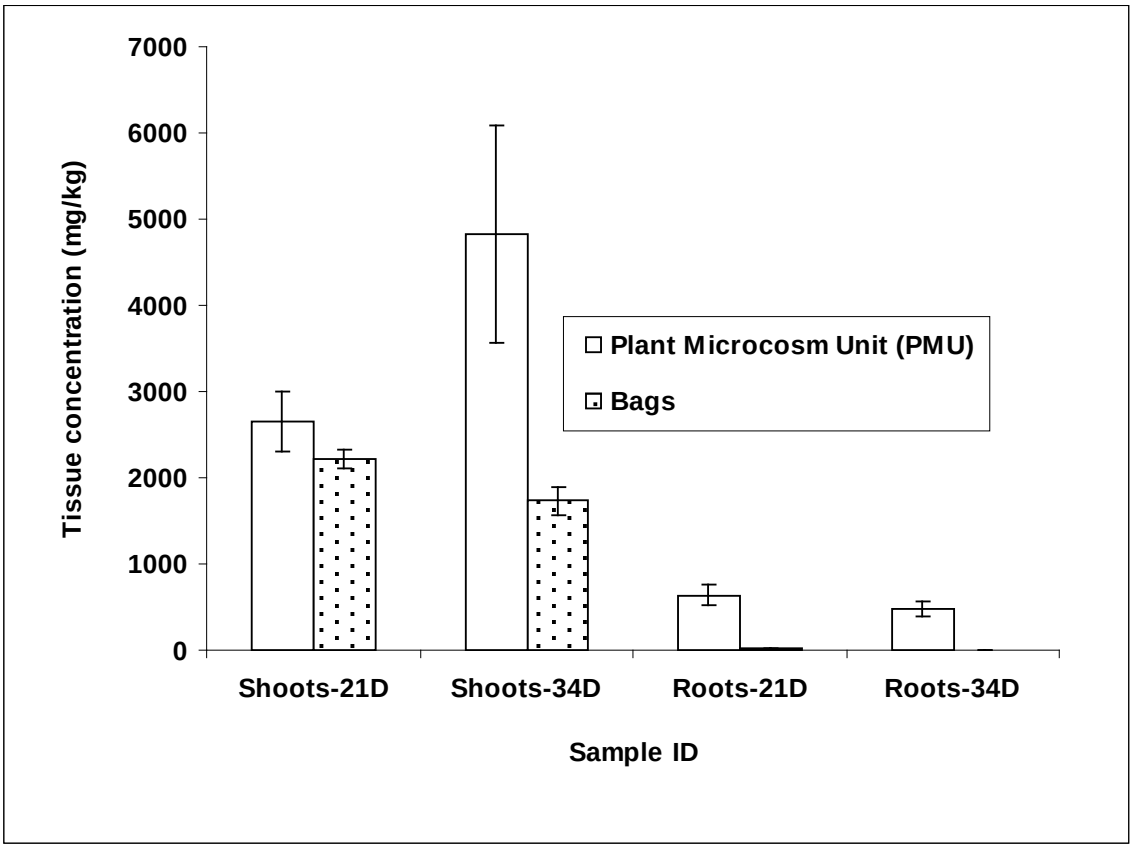


Figure 70. Accumulation of RDX in ryegrass (shoots or roots) exposed to 30 mg RDX/kg Sassafras sandy loam soil in different test systems. Results are expressed as means and standard errors (n=3).

9.1.7.2. Use of the plant accumulation microcosm unit (PAM) for assessing RDX accumulation in plants

Results showed that RDX is rapidly accumulated in both shoots and roots with respect to the exposure period (Figure 71). A plateau or a steady state (based on at least two exposure periods showing similar accumulation) was reached after 21 d. The BCF values for shoots ranged from 22 to 77 depending on the exposure time or concentrations (Table 82). These data are in agreement with the results of studies by Rocheleau *et al.* (2008), in which the ryegrass shoots-based BCF values decreased with increasing soil concentrations. However, considerably greater amounts of RDX in the ryegrass shoots were measured in the present study. For example, Rocheleau *et al.* (2008) recovered 804 mg RDX/ kg dry tissue following a 42-d exposure at 91 mg/kg compared to 2031 mg RDX/kg dry tissue determined in the present study after 34 d using a similar soil concentration (Figure 71). Procedural differences between the two studies, e.g., use of the plant accumulation microcosm unit instead of plastic bags, the dissimilar approaches to watering of plants, could contribute to these differences. Concentrations of RDX in root extracts increased with increasing concentrations in soil, and were not affected by the duration of

exposure. The maximum RDX concentration in roots of 237 mg/kg was measured in the 100 mg/kg soil treatment after 21 d. The root-based BCF values for RDX were between 2 and 6.

In conclusion, most of the accumulated RDX was found in ryegrass leaves and lesser amount of RDX was found in roots. The passage of the chemical through the plant roots and translocation to the shoots was characterized further by determining the translocation factor (TF). The TF was calculated by dividing the shoots-based BCF value by the roots-based BCF value. Average TF values were 9, 13, and 10 in the 10, 30, and 100 mg/kg soil RDX treatments, respectively.

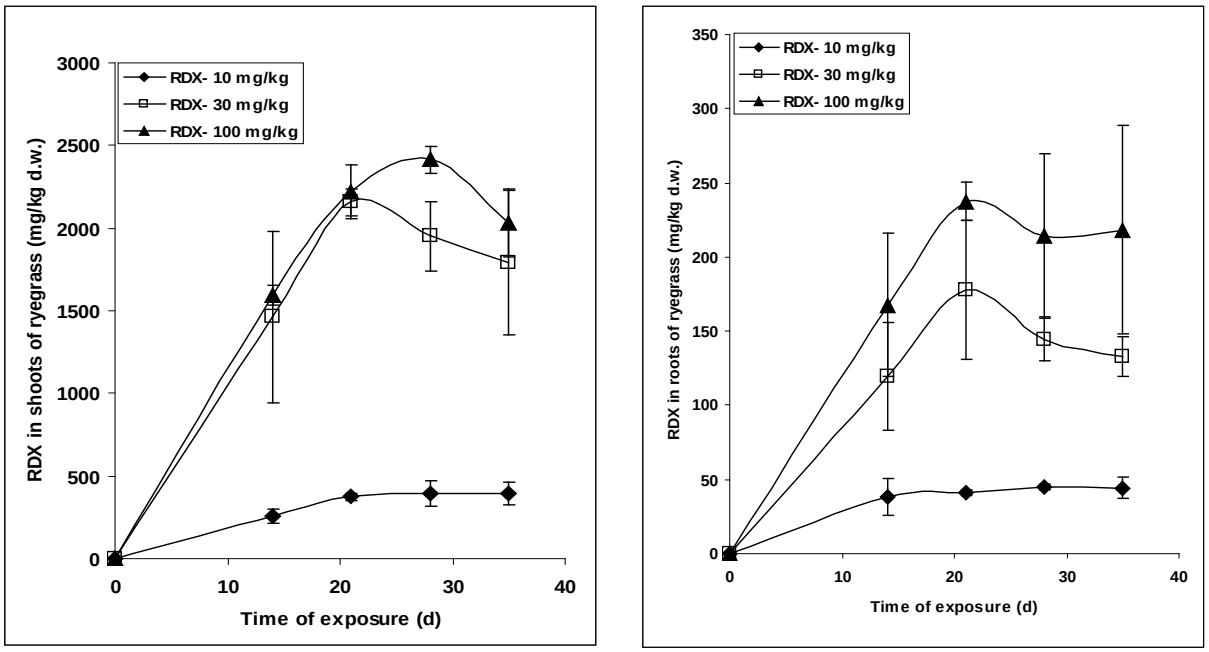


Figure 71. Accumulation of RDX in shoots and roots of ryegrass.

Table 82. Parameters measured for the accumulation of RDX in ryegrass species following exposure to amended soil.

Nominal concentrations of RDX (mg/kg)	Exposure time (d)	Shoots* (mg/kg) Quantity in tissue (mg/kg)	BCF	Roots* (mg/kg) Quantity in tissue (mg/kg)	BCF	TF
10	14	256.3 (41.4)	29.8	38.4 (12.1)	4.5	6.7
	21	373.4 (18.1)	43.8	41.1 (1.8)	4.8	9.1
	28	393.6 (79.7)	53.6	44.7 (2.0)	6.1	8.8
	34	395.7 (69.1)	57.4	44.3 (7.3)	6.4	9.9
30	14	1461.5 (519.9)	49.1	119.7 (36.3)	4.0	12.2
	21	2156.2 (83.8)	77.4	178.1 (46.8)	6.4	12.1
	28	1951.2 (209.0)	76.2	144.8 (14.5)	5.7	13.5
	34	1790.0 (434.6)	75.7	133.2 (13.5)	5.6	13.5
100	14	1594.0 (59.8)	16.5	167.6 (48.1)	1.7	9.5
	21	2218.5 (164.4)	23.0	237.5 (13.1)	2.5	9.3
	28	2414.2 (100.0)	25.1	214.3 (55.8)	2.2	11.3
	34	2031.5 (207.0)	22.3	218.4 (70.4)	2.4	9.3

Table notes: * Data are expressed as mean with standard deviation in brackets, n=3.
Bioconcentration factor, BCF calculated as the ratio of quantity in tissue to soil.
Translocation factor, TF calculated as the ratio of shoots to roots BCF values.

9.1.7.3. Mass balance studies of ^{14}C-RDX uptake by ryegrass

This feasibility study showed a good recovery of radioactivity (dpm) in the 21-d samples (from 95 to 111% of total radioactivity added at the start of the test, T_0), and in the 34-d samples (100 to 117%). Table 83 shows the results of the mass balance studies. Data in this table are presented as percentages of total radioactivity added following the 21, or 34-d exposure to ^{14}C-RDX. Most (at least 91.5%) of the radioactivity remained in soil. Only 2.6 to 7.4% was found in shoots, and 1.0% were recovered from the alkali traps. These range-finding data indicate that the PAM can be used for the plant accumulation studies using ^{14}C-energetic materials.

Table 83. Mass balance for ^{14}C-RDX based on recovery of ^{14}C-activity in plant microcosm units housing plants exposed for 21, or 34 days to ^{14}C-RDX in amended Sassafras sandy loam soil.

Sample analyzed	Recovery (%) after soil exposure					
	21 d			34 d		
Soil	96.29	±	0.55	91.53	±	0.96
Shoots	2.59	±	0.54	7.41	±	0.86
Roots	0.08	±	0.02	0.10	±	0.02
Mineralized	1.03	±	0.02	0.96	±	0.10

Based on the results of non-labeled studies, 30 mg/kg of RDX was selected as concentration to test with a labeled RDX. Results showed presence of radioactivity in both shoots and roots of ryegrass as early as 14 d following exposure to amended soil. In addition, the radioactivity increased in both matrices until it reach a plateau after 28 d (Figure 72). Shoots always have more radioactivity than roots samples. Table 84 shows that radio-labeled compounds are accumulated in both shoots and roots of ryegrass. The BCF calculated for shoots vary from 101 to 175 compared to 49 to 75 found with non-labeled material (Table 82). In addition considerably high level of radioactivity was observed in roots ($10.9x10^{+5}$ dpm/g) after 34 d, BCF calculated for roots were from 30.5 to 75 compared to 4.0 to 6.4 in non labeled studies. The discrepancy with labeled and non-labeled studies could be attributed to transformation of RDX into other carbon-based products that were not extracted from tissue in the non-labeled experiments. Additional studies are required to confirm this assumption.

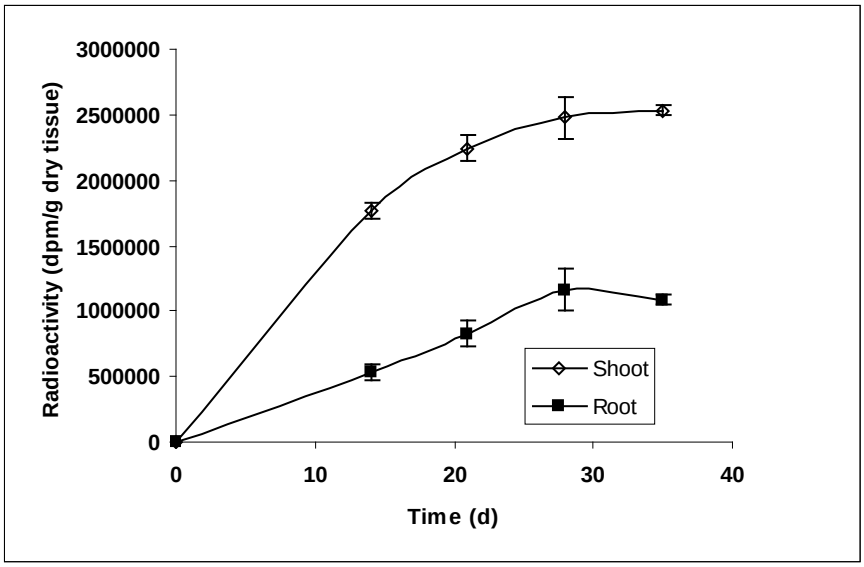


Figure 72. Time-dependent increase in the ^{14}C-radioactivity measured in roots and shoots tissue of ryegrass following exposure to ^{14}C-RDX in amended soil (30 mg/kg).

Table 84. Summary of the parameters measured for the accumulation of ^{14}C-RDX in ryegrass following exposure in amended soil.

Parameter	Radioactivity measured in soil or tissue (10^5 dpm/g)				
	Start	14 d exposure	21 d exposure	28 d exposure	34 d exposure
Soil	1.79 (0.05)	1.75 (0.05)	1.68 (0.09)	1.56 (0.02)	1.45 (0.05)
Shoots		17.66 (0.63)	22.45 (1.00)	24.77 (1.61)	25.3 (0.40)
Roots		5.31 (0.82)	8.24 (0.61)	11.65 (0.90)	10.88 (0.85)
Bioconcentration of ^{14}C-RDX and its metabolites in plants					
BCF shoots		101.2	133.3	158.9	174.6
BCF roots		30.5	48.9	74.7	75.0
TF		3	3	2	2

Table notes: Data are expressed as mean of triplicate data with standard deviation in brackets.
BCF was calculated as the ratio of tissue to soil at the corresponding exposure time for either shoots or roots.

9.2. Bioaccumulation of TNT

9.2.1. Uptake of non-labeled TNT in earthworms

Test was conducted to determine the best soil moisture equilibration time necessary to perform accumulation studies with TNT. Results showed that TNT decreased in soil as early as 3 h following the addition of water (Table 85). Both 2-ADNT and 4-ADNT were present at low concentrations throughout the study. Recoveries of TNT from soil were 94, 92, and 67 percent of the initial TNT concentration at 0 h after 3 h, 1 d, and 4 d from addition of water, respectively. The combined quantities of 2-ADNT and 4-ADNT formed in soil corresponded to 0.2, 0.4, and 7 percent of the initial TNT concentration at 0 h after 3 h, 1 d, and 7 d from hydration, respectively.

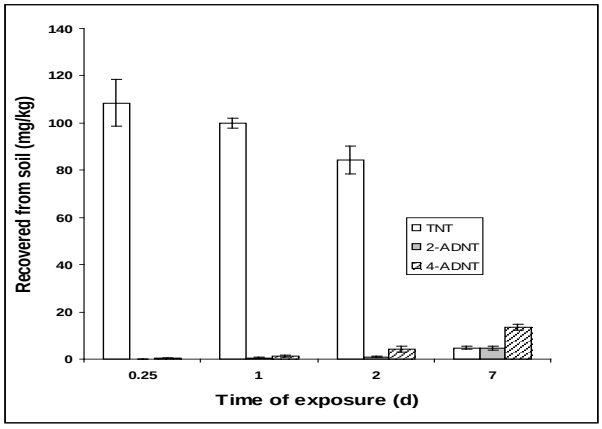
Table 85. Analyses of soil following the different moisture equilibration treatments of TNT-amended soil prior to exposure of earthworms.

Moisture equilibration time (d)	Compound recovered from soil following exposure to TNT-amended soil		
	TNT (mg/kg)	**2-ADNT (mg/kg)**	**4-ADNT (mg/kg)**
0.00	124.10 (1.73)	ND	ND
0.13	116.61 (1.02)	0.05 (0.07)	0.17 (0.06)
1.00	114.66 (3.70)	0.16 (0.01)	0.29 (0.25)
4.00	83.66 (5.68)	1.28 (0.44)	4.60 (0.21)

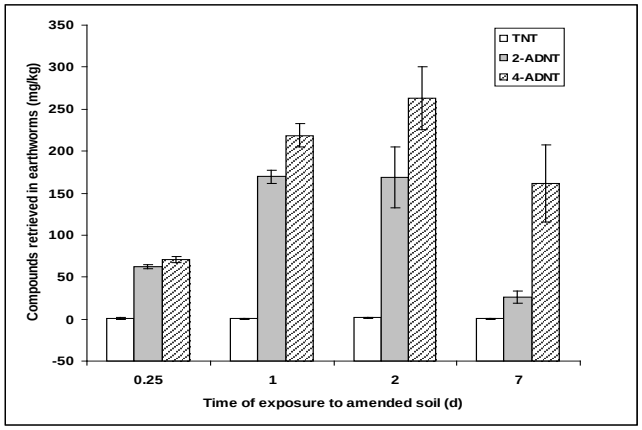
Table notes: ND: Not detected (detection limit = 0.05 mg/kg). Data expressed as mean of triplicate values with standard deviation in brackets.

Concentrations of TNT in soil extracts showed similar trends in the presence, or absence of earthworms. A time-dependent decrease of TNT concentrations in soil was observed (68 to 110 mg/kg TNT at 6h experiment time compared to 4.7 -6.2 mg/kg TNT at 7 d) (Table 86). In addition, 2-ADNT and 4-ADNT were recovered in soil (maximum concentration was 12.2 mg/kg for 2ADNT and 13.5 mg/kg for 4ADNT (Table 86). TNT extracted from the earthworm tissues was very low compared to RDX found in tissue following exposure in soil. The maximum amount of TNT (2.4 mg/kg) was extracted from the earthworms exposed for 6 h in soil that was allowed to moisture-equilibrate for 1 d prior to addition of the earthworms (Table 86 and Figure 73). Both 2-ADNT and 4-ADNT were found in earthworms exposed to TNT-amended soil in all moisture-equilibration treatments. No significant ($p>0.05$) difference in uptake of combined quantities of 2-ADNT and 4-ADNT by the earthworms were found between the 3-h and 1-d moisture-equilibration periods for any of the earthworm exposure durations tested. The amount of 2-ADNT and 4-ADNT extracted from the earthworms exposed in soil that was moisture-equilibrated for 4 d prior to addition of the earthworms was lower than that in the earthworms exposed in soil that was moisture-equilibrated for 3 h or 1 d prior to addition of the earthworms. The t-test showed a significant ($p<0.05$) difference between the 3-h and 4-d moisture-equilibration periods, and no significant ($p>0.05$) differences between the 3-h and 1-d moisture-equilibration periods. Based on these results, TNT-amended soil was moisture-equilibrated for 3 h or 1 d prior to addition of earthworms in the subsequent studies.

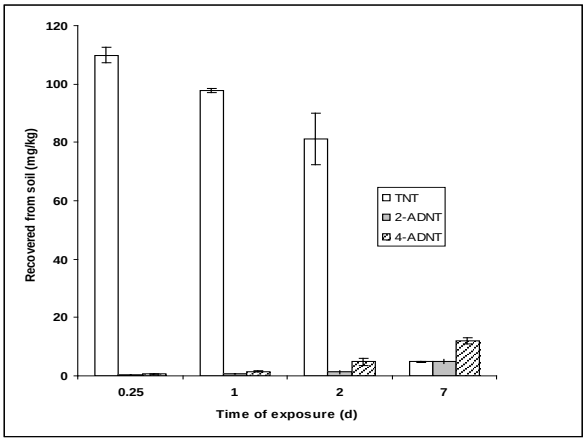
Moisture-equilibration 0.13 d



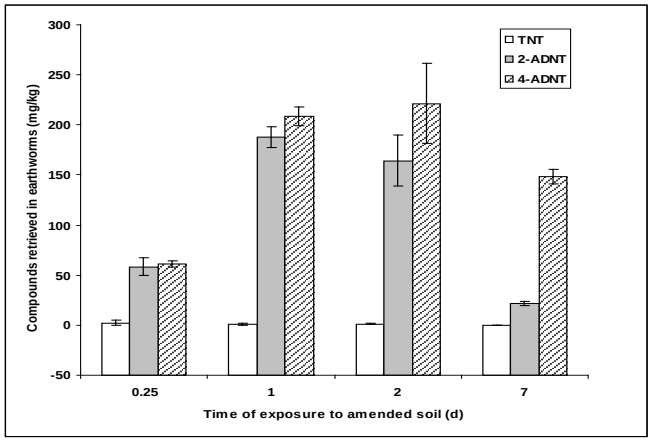
Moisture-equilibration 0.13 d



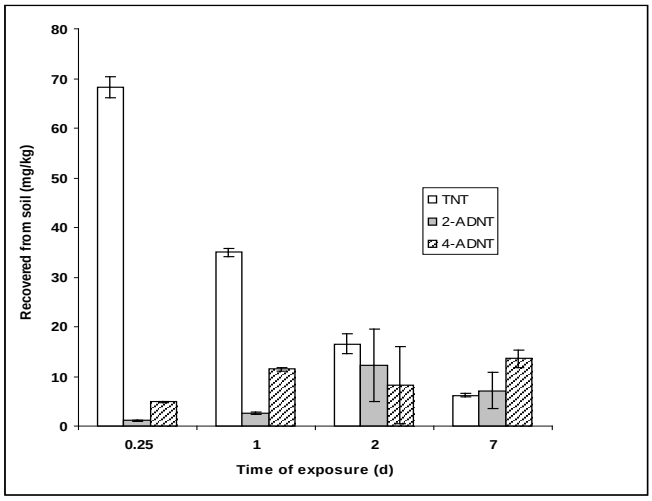
Moisture-equilibration 1 d



Moisture-equilibration 1 d



Moisture-equilibration 4 d



Moisture-equilibration 4 d

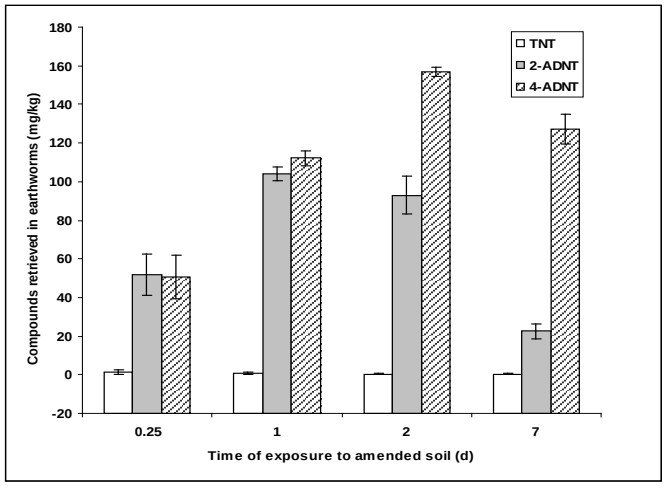


Figure 73. TNT and its metabolites recovered from soil and earthworms following exposure to TNT-amended soil.

Table 86. Analyses of soil and earthworms tissues following exposure to TNT-amended soil with different moisture equilibration treatments.

Moisture equilibration time (d)	6 h			1 d			2 d			7 d		
	Recovered from soil (mg/kg)											
	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT
0.13	108.49 (9.82)	0.08 (0.07)	0.35 (0.12)	99.87 (2.14)	0.61 (0.16)	1.26 (0.31)	84.23 (5.85)	1.04 (0.19)	4.12 (1.24)	4.71 (0.57)	4.76 (0.88)	13.36 (1.30)
1	**109.93 (2.67)**	0.18 (0.03)	0.62 (0.13)	97.80 (0.81)	0.71 (0.02)	1.51 (0.20)	81.03 (8.84)	1.26 (0.42)	4.79 (1.38)	4.90 (0.19)	5.08 (0.71)	12.12 (1.10)
4	68.25 (2.05)	1.09 (0.05)	4.85 (0.10)	35.00 (0.88)	2.54 (0.21)	11.44 (0.42)	16.58 (2.03)	**12.24 (7.25)**	8.21 (7.70)	6.20 (0.33)	7.12 (3.68)	**13.53 (1.73)**
Moisture equilibration time (d)	**In earthworm tissues (mg/kg)**											
	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT
0.13	0.55 (0.96)	62.35 (2.54)	70.56 (3.38)	0.42 (0.73)	169.69 (7.76)	218.85 (13.80)	1.51 (0.33)	168.55 (36.32)	**263.24 (37.17)**	0.33 (0.36)	26.12 (6.91)	160.99 (46.03)
1	**2.41 (2.53)**	58.22 (8.76)	61.27 (2.77)	1.24 (0.94)	**187.62 (10.23)**	208.79 (9.12)	1.30 (0.82)	164.22 (25.44)	221.41 (40.13)	ND	21.89 (2.19)	148.40 (7.10)
4	1.14 (2.19)	51.79 (10.68)	50.62 (11.33)	0.68 (0.70)	104.03 (3.78)	112.11 (3.75)	0.29 (0.27)	93.01 (9.94)	157.00 (2.32)	0.35 (0.31)	22.45 (4.03)	126.94 (7.73)

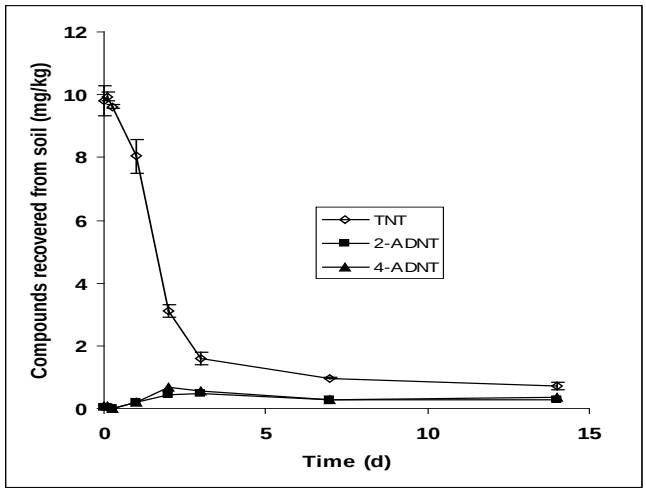
Moisture equilibration time (d)	6 h			1 d			2 d			7 d		
	Recovered from soil (mg/kg)											
	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT
0.13	108.49 (9.82)	0.08 (0.07)	0.35 (0.12)	99.87 (2.14)	0.61 (0.16)	1.26 (0.31)	84.23 (5.85)	1.04 (0.19)	4.12 (1.24)	4.71 (0.57)	4.76 (0.88)	13.36 (1.30)
1	**109.93 (2.67)**	0.18 (0.03)	0.62 (0.13)	97.80 (0.81)	0.71 (0.02)	1.51 (0.20)	81.03 (8.84)	1.26 (0.42)	4.79 (1.38)	4.90 (0.19)	5.08 (0.71)	12.12 (1.10)
4	68.25 (2.05)	1.09 (0.05)	4.85 (0.10)	35.00 (0.88)	2.54 (0.21)	11.44 (0.42)	16.58 (2.03)	**12.24 (7.25)**	8.21 (7.70)	6.20 (0.33)	7.12 (3.68)	**13.53 (1.73)**
Moisture equilibration time (d)	**In earthworm tissues (mg/kg)**											
	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT	TNT	2-ADNT	4-ADNT
0.13	0.55 (0.96)	62.35 (2.54)	70.56 (3.38)	0.42 (0.73)	169.69 (7.76)	218.85 (13.80)	1.51 (0.33)	168.55 (36.32)	**263.24 (37.17)**	0.33 (0.36)	26.12 (6.91)	160.99 (46.03)
1	**2.41 (2.53)**	58.22 (8.76)	61.27 (2.77)	1.24 (0.94)	**187.62 (10.23)**	208.79 (9.12)	1.30 (0.82)	164.22 (25.44)	221.41 (40.13)	ND	21.89 (2.19)	148.40 (7.10)
4	1.14 (2.19)	51.79 (10.68)	50.62 (11.33)	0.68 (0.70)	104.03 (3.78)	112.11 (3.75)	0.29 (0.27)	93.01 (9.94)	157.00 (2.32)	0.35 (0.31)	22.45 (4.03)	126.94 (7.73)

Table notes: Bold values represent the maximum amount of chemical found in soil or tissue for either TNT or its metabolites. Data expressed as mean of triplicate with standard deviation in brackets. ND: not detected (detection limit = 0.05 mg/kg in soil and 4 mg/kg in earthworm tissues).

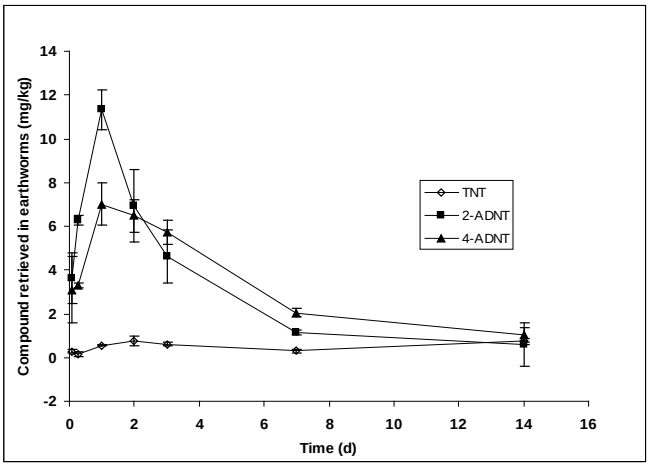
Earthworms were then exposed to different concentrations of TNT-amended soil in order to better understand the accumulation profiles of this compound. Results showed that the concentration of TNT in soil decreased as the exposure time increased (Figure 73, Table 86). In addition, the two metabolites of TNT, 2-ADNT and 4-ADNT, were present. The amount of these metabolites in soil reached a maximum after one day, and then decreased after the 2, 3, and 7–d exposure periods in the 10, 50, and 100 mg/kg soil treatments, respectively (Figure 74).

Low concentrations (less than 2 mg/kg) of TNT were recovered from the earthworm tissues after exposure to amended soil up to and including 14 d (Figure 74). The maximum amount of TNT (1.83 μg/g dry tissue) was recovered from the earthworms exposed for 2 h in soil amended with 100 mg TNT/kg dry soil. Both 2-ADNT and 4-ADNT were found in all earthworms exposed in TNT-amended soil throughout the assay. The maximum amounts of 2-ADNT and 4-ADNT (174 and 229 μg/g dry tissue, respectively) were recovered from tissue of earthworms exposed to TNT-amended soil at 100 mg/kg for 3 d. These data indicate that the TNT metabolites in earthworms could either originate from the soil or result from TNT transformation by the earthworms. This study suggests that TNT was metabolized and did not accumulate in earthworm tissues.

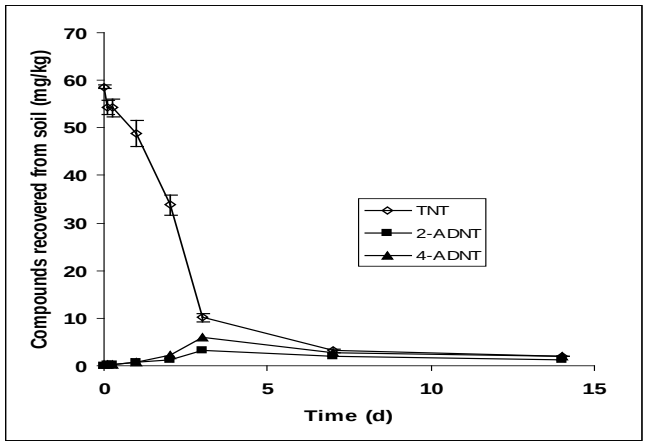
A: Soil amended with 10 mg/kg TNT



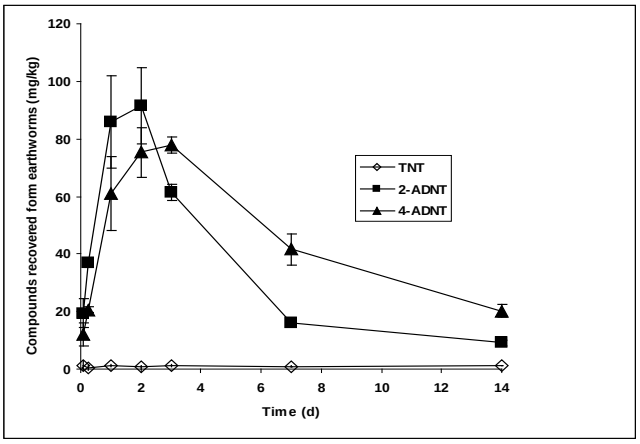
D: Earthworm exposed to soil amended with 10 mg/kg TNT



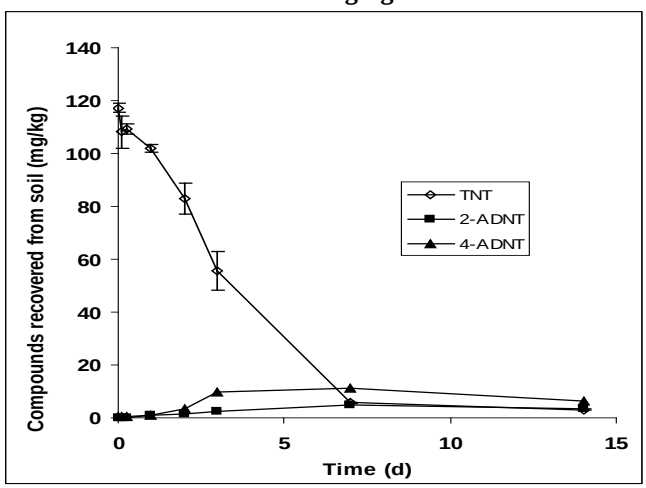
B: Soil amended with 50 mg/kg TNT



E: Earthworm exposed to soil amended with 50 mg/kg TNT



C: In soil amended with 100 mg/kg TNT



F: Earthworm exposed to soil amended with 100 mg/kg TNT

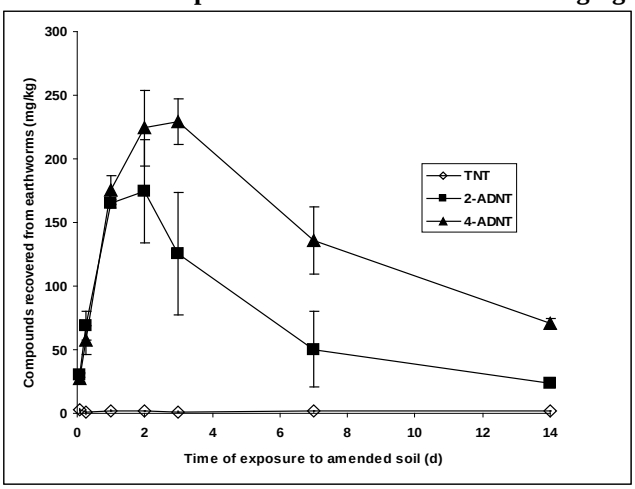


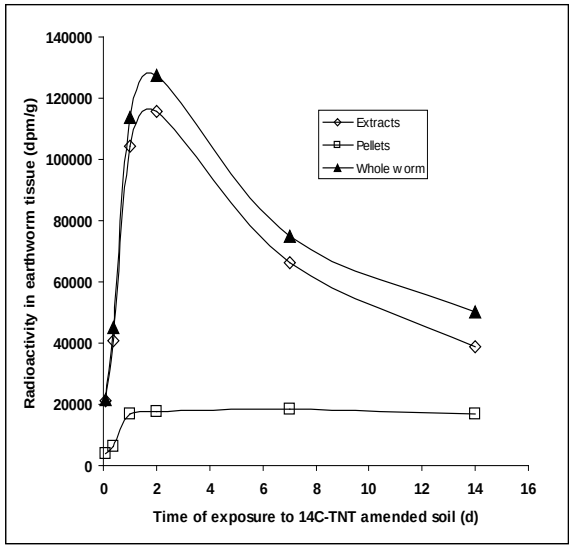
Figure 74. TNT and its metabolites found in soil, and in earthworms following exposure to TNT-amended soil.

9.2.2. Uptake of ^{14}C-TNT in earthworms

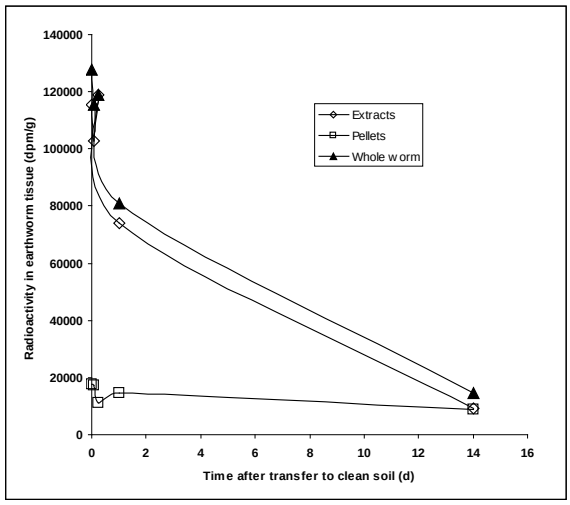
^{14}C-TNT was used to identify the potential metabolites which were not measured in studies using unlabeled compounds. Soil extracts at day 0 showed that the soil TNT concentration was 100 mg/kg. At day 14, mortality was observed in a few of the TNT treatment vessels. Analyses of soil extracts showed that radioactivity measured in soil samples ranged between 92 to 105% of the amount at day 0. Therefore, a small quantity of TNT (up to 8% of the total use at day 0) was taken up by the earthworms.

Results also showed that the maximum amount of metabolites in the earthworms was reached after 2 to 3 d of exposure in soil. The results of these studies are presented in Figure 75, and showed that radioactivity measured in soil before introducing the earthworms was 31,250 dpm/g dry soil. This radioactivity remained in the soil until the end of the uptake phase (recovery greater than or equal to 85.6 percent). Radioactivity was also measured in earthworm tissues (acetonitrile-extractable and non-extractable portions) following exposure to ^{14}C-TNT amended soil. Radioactivity in tissue (extractable portion) increased to 115,580 dpm/g after 2 d, and then decreased to 38,806 dpm/d by the end of the 14-d uptake phase. Radioactivity in residues of extracted tissue (non-extractable portion) reached a plateau after 1 d. The radioactivity in the earthworm tissue was eliminated slowly after transfer to the non-amended soil. Eight to 21 percent of the original amount of radioactivity in the earthworm extract (extractable portion) and 39 to 49 percent of the original amount of radioactivity in the earthworm pellet (non–extractable portion) remained by the end of the 14-d elimination phase. HPLC analyses of the earthworm tissue extract revealed the presence of metabolites, 2-ADNT and 4-ADNT. The parent compound TNT was not found in any of the tissue samples tested. This study confirmed that TNT does not accumulate in the earthworms following exposure in ^{14}C-TNT amended soil.

Uptake 14 d



Elimination following 2 d uptake



Elimination following 14 d uptake

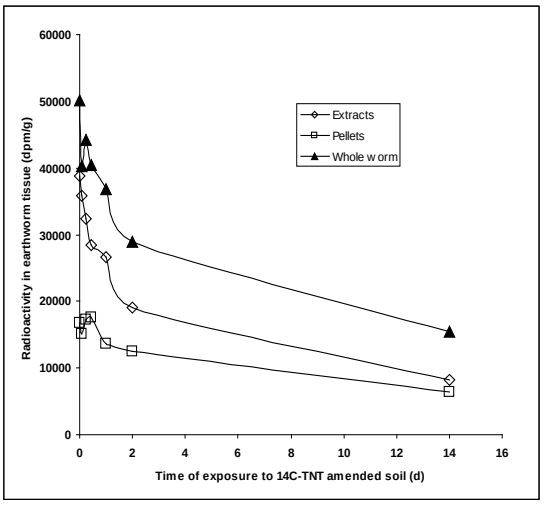


Figure 75. Follow up of TNT and its metabolites in earthworms following 2 d or 14 d exposure in amended soil.

9.2.3. <u>Uptake of non-labeled TNT in plants</u>

A study was initiated to determine the uptake of TNT by ryegrass in SSL2007d soil using nominal TNT exposure concentrations of 10, 30, 100, and 150 mg/kg. Similar to the results of the earthworm studies, TNT decreased in soil with increasing exposure period for soil amended with 100 mg/kg or higher. Very low amount of TNT (maximum 0.45 mg/kg) was recovered from soil amended with 10 mg/kg (Table 87).

Table 87. TNT recovered from amended soil during the ryegrass studies

Nominal concentration of TNT (mg/kg)	TNT measured in soil after growth of ryegrass (mg/kg)			
	14 d	**21 d**	**28 d**	**35 d**
10	0.45 (0.02)	0.39 (0.05)	0.32 (0.02)	0.36 (0.02)
30	1.18 (0.18)	0.98 (0.08)	0.76 (0.05)	0.75 (0.10)
100	5.98 (0.59)	3.70 (0.24)	2.67 (0.41)	2.31 (0.06)
150	19.30 (0.82)	11.77 (2.47)	5.58 (0.55)	4.43 (0.39)

Table notes: Data are expressed as mean with standard deviation in brackets, n=3.

At each time period (14, 21, 28, and 35 d), an attempt to calculate BCF was done by dividing the measured concentration of TNT in tissue (when present) by the measured concentration of TNT in soil. Results showed that TNT is not accumulated in shoots of ryegrass (data not shown). TNT found in roots of ryegrass (Figure 76) was close to the detection limit in tissue. A maximum BCF value of 0.75 was determined in ryegrass roots after 21 d at 150 mg/kg nominal soil TNT concentrations. In addition, 2-ADNT and 4-ADNT were found in both shoots and roots of ryegrass (Figure 76). These results suggest that ryegrass may transform TNT in roots into 2-ADNT and 4-ADNT, which are further translocated to shoots.

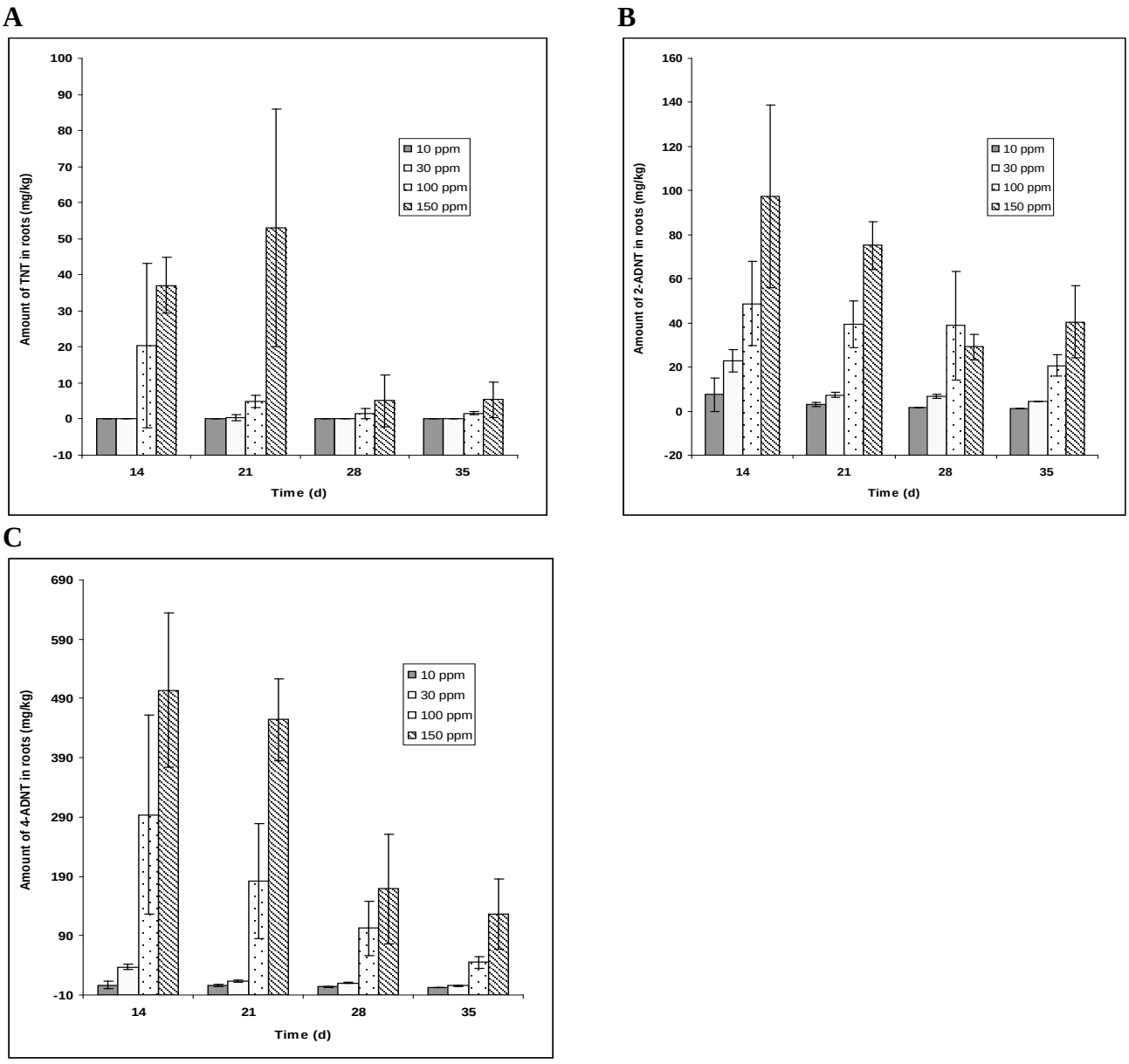


Figure 76. Time course studies of compounds measured in plant tissue following exposure to TNT amended soil.

9.2.4. Uptake of ^{14}C-TNT in plants

Following exposure studies to non-labeled material, uptake and mass balance experiments were conducted using ryegrass seeded in soil amended with ^{14}C-TNT (100 mg/kg) for up to 35 d. Most of the radioactivity remained in the soil. The roots had more radioactivity than the roots (Figure 77). Results also showed that radioactivity was recovered at 90% or higher at the end of

each exposure periods. The distribution of radioactivity based on total recovery in soil, mineralized, shoots and roots are reported in Table 88.

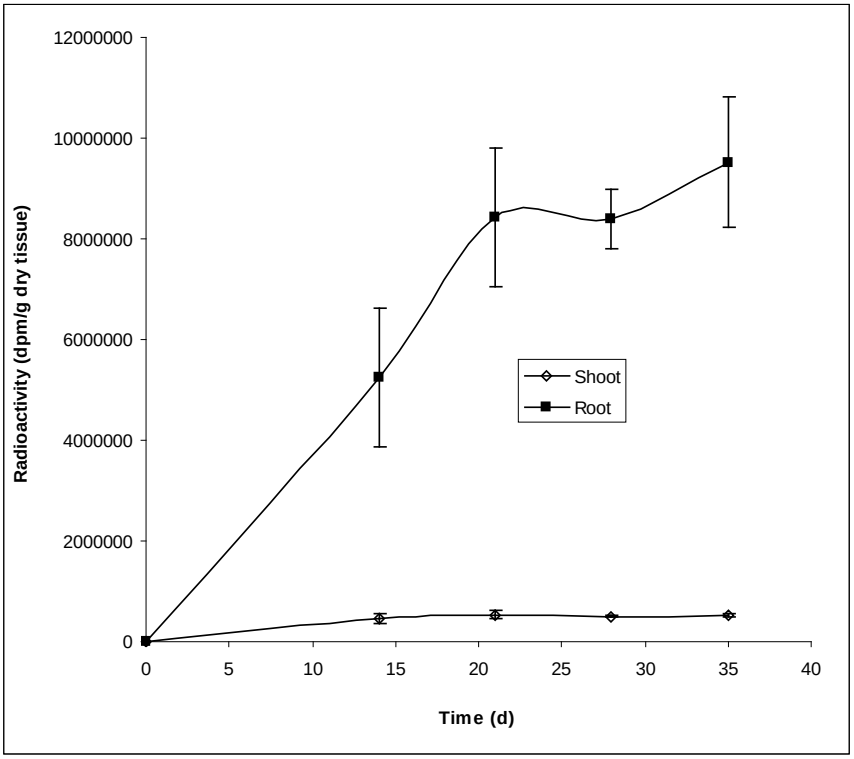


Figure 77. Radioactivity measured in plant tissue following exposure to ^{14}C-TNT amended soil.

Table 88. Distribution of the radioactivity between the soil, plant tissue, and mineralization.

Exposure period (d)	Recovery in soil (%)	Recovery in shoots (%)	Recovery in roots (%)	Recovery as CO_2 (%)
14	99.73	0.08	0.07	0.12
21	99.38	0.14	0.31	0.17
28	99.07	0.23	0.49	0.21
35	99.09	0.25	0.39	0.28

9.3. Bioaccumulation of HMX

9.3.1. Uptake of non- labeled HMX in earthworms

Studies with SSL soil were carried out to determine if the kinetic-based approach used in RDX accumulation studies with earthworms could be also applied to similar studies with HMX. Exposure concentration of HMX was selected based on the results of previous studies (Robidoux *et al*. 2001b, 2002a). Tissue and soil samples were collected according to procedures described for the RDX bioaccumulation test. These samples were analyzed using the USEPA Method 8330A. HMX concentrations in tissue and soil samples were expressed as mg/kg dry tissue and mg/kg dry soil, respectively.

The greatest uptake of HMX in tissue (20 mg/kg) occurred after 2 d exposure to soil previously moisture-equilibrated for one-day (Figure 78).In addition, HMX concentrations in tissue were 13.7 and 13.5 mg/kg after 4 and 7 d of moisture-equilibration, respectively. These range-finding results indicate that the same amount of HMX was available for the earthworms at 4 and 7 d. Therefore, a steady state condition for HMX-amended SSL soil can be attained after 4 d from soil hydration.

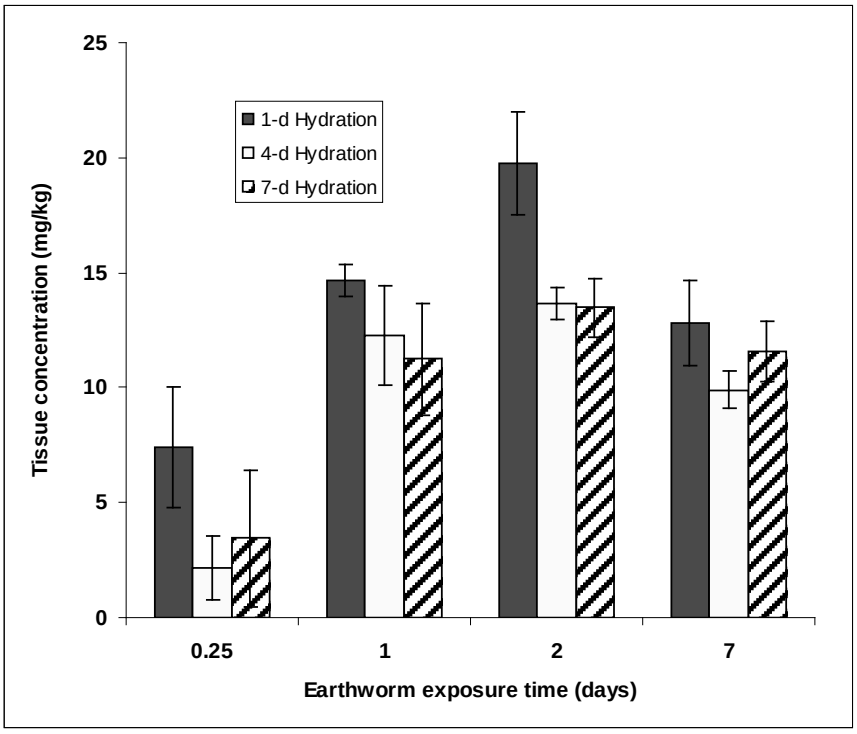
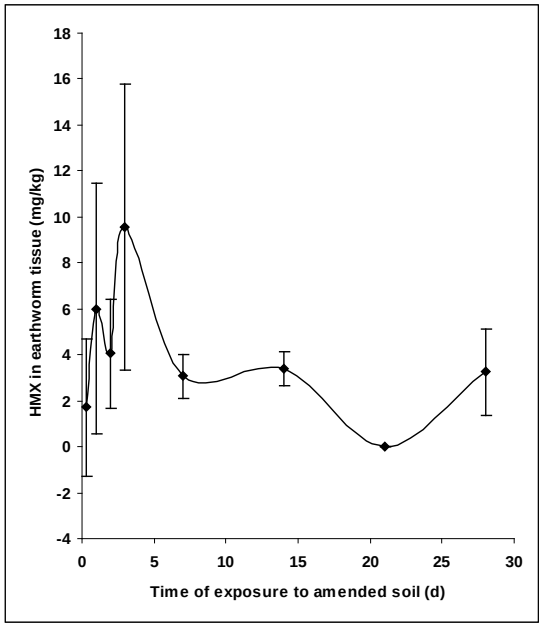


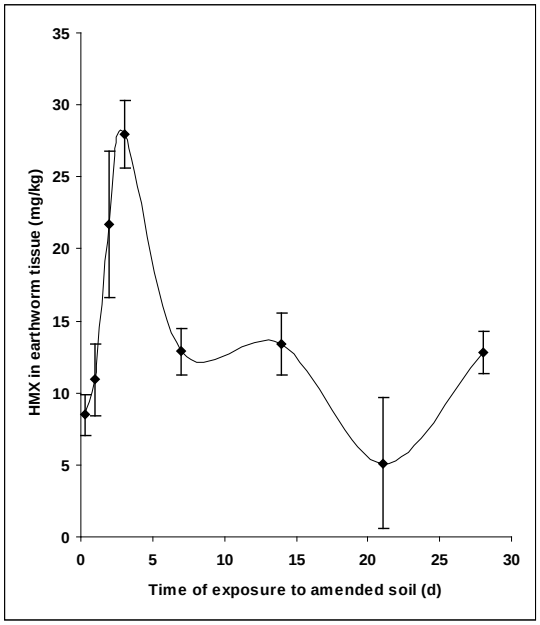
Figure 78. Concentration of HMX in *Eisenia andrei* exposed to nominal 100 mg HMX/kg Sassafras sandy loam soil for 7 d following different moisture-equilibration periods from the initial soil hydration. Results are expressed as means and standard errors (n=3).

Results showed low (2.3 mg HMX/kg dry tissue) or non-detectable uptake of HMX by earthworms exposed to 1 mg HMX/kg dry soil (data not shown). HMX uptake increased with increasing concentrations in soil for all selected exposure durations (Figure 79). The maximum uptake (1950 mg HMX/kg dry tissue) was determined at 10000 mg HMX/kg dry soil. It occurred after the two-day exposure period and was followed by a decrease in HMX concentration until the end of the 28-d study. Similar results were determined for 10, 100, and 1000 mg HMX/kg dry soil treatments. These results suggest that HMX can either be excreted or sequestered in the earthworm tissues.

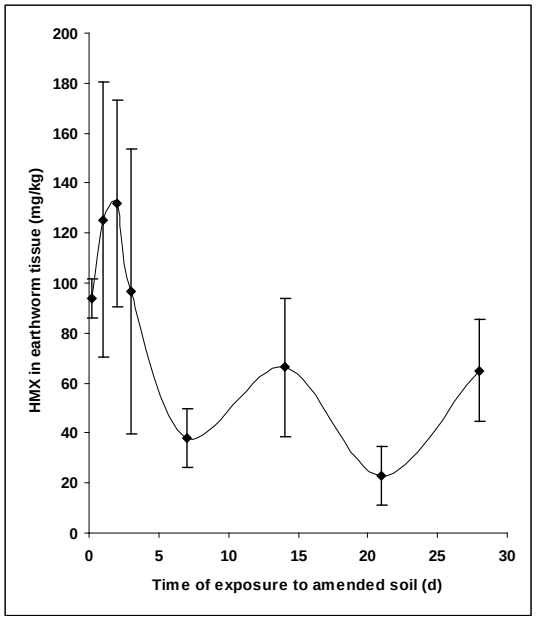
Exposure to 10 mg/kg in soil



Exposure to 100 mg/kg in soil



Exposure to 1,000 mg/kg in soil



Exposure to 10,000 mg/kg in soil

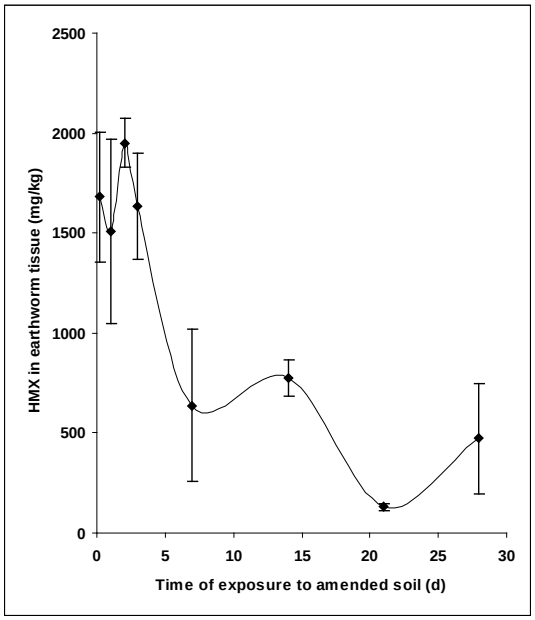


Figure 79. HMX is taken up by earthworm and is dependent on the amended soil concentrations.

9.3.2. Uptake of ^{14}C-HMX in earthworms

A kinetic soil bioaccumulation test was conducted using ^{14}C-HMX amended in SSL2007d soil. Determination of uptake and elimination kinetics of HMX in earthworms was done using an adaptation of the draft OECD Guideline (OECD, 2010). The level of radioactivity measured in soil, trapped as $^{14}C\text{-}CO_2$ or found in earthworms, is reported in the Figure 80. Results showed that radioactivity slightly decrease or remained stable in soil until the 28-d uptake study. Only 2% or less was recovered in earthworms or as CO_2 (Figure 80).

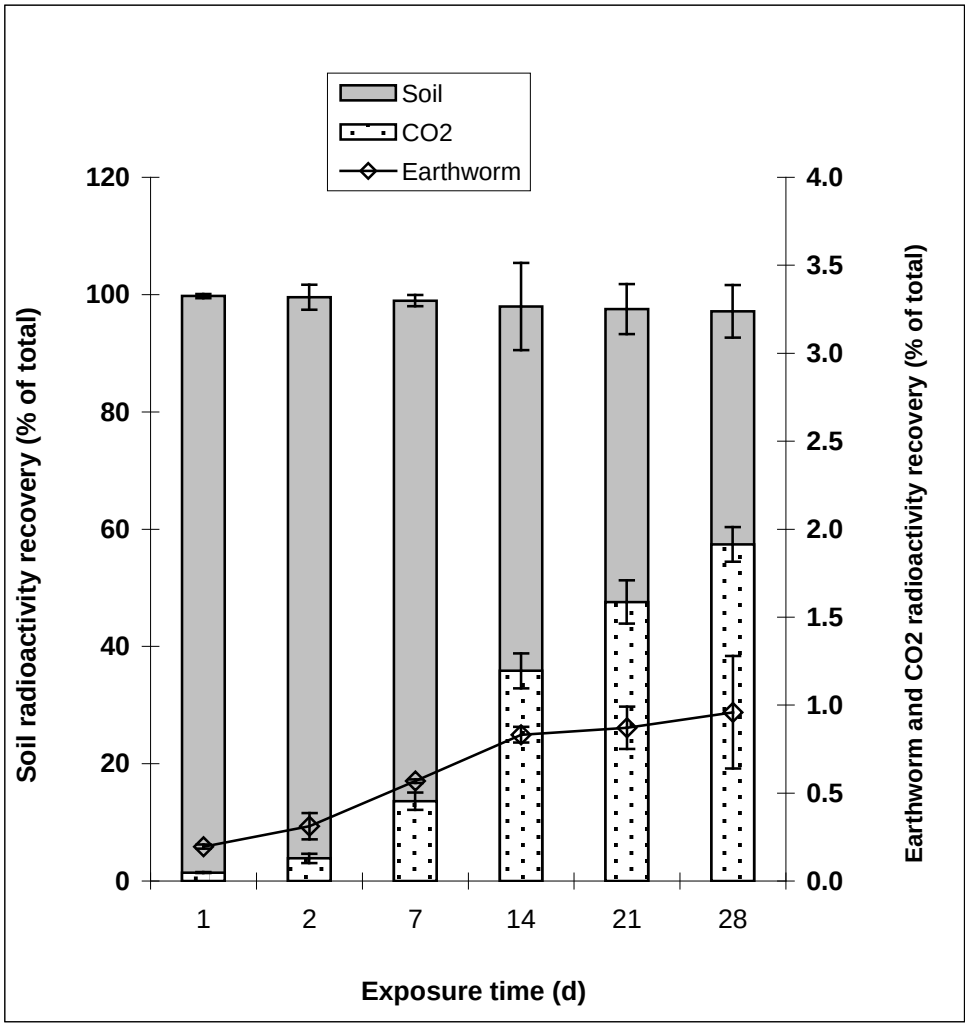


Figure 80. Radioactivity recovered in soil, earthworm and as CO_2 following exposure of earthworms to ^{14}C-HMX amended soil.

Analyses of earthworms tissues showed that the ^{14}C- HMX or its metabolites increased in earthworms with respect to the exposure period (Figure 81a). A maximum uptake (7989 dpm/g dry tissue) was observed in combusted whole earthworm tissues, after 28 d exposure to 10 mg/kg of labeled compound. However, only 45% of the total radioactivity (4300 dpm/g) was recovered

in the extractable fraction, whereas 55% (5280 dpm/g) was measured in the pellet (non-extractable fraction). In addition radioactivity measured in worm extract was identified as HMX in HPLC, and no metabolites was found in the extracts (Figure 81b). Extracts from the earthworm tissues collected in the elimination phase showed that the radioactivity decreased to near zero after 28 d in the non-amended soil (Figure 81a). However, 2460 dpm/g remained in the pellet after the 28-d elimination phase. This non-extractable portion corresponds to 46% of the maximum radioactivity measured after the 28-d uptake period, thus suggesting that HMX may be bound to cellular macromolecules in the earthworm tissues, and that HMX has a lower potential for accumulation in the earthworms compared with RDX.

A: Combustion quantification **B: HPLC quantification**

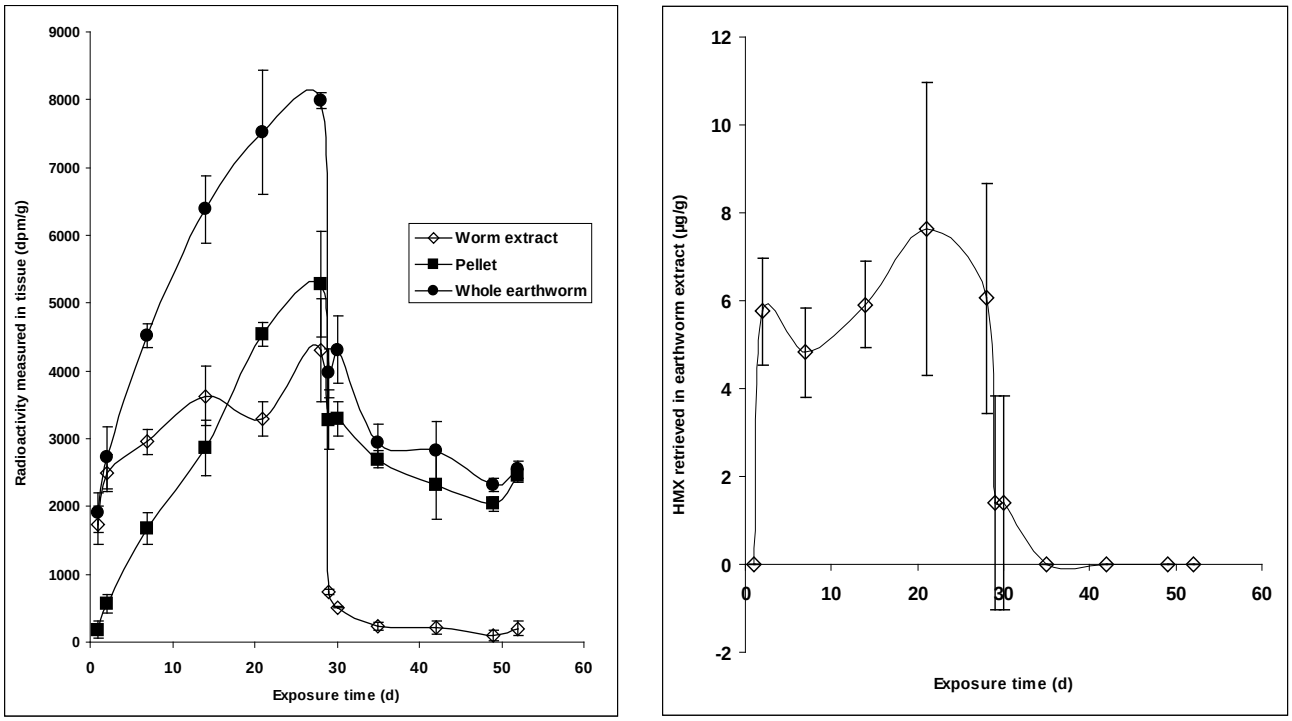


Figure 81. Radioactivity found in tissue following exposure of earthworms to ^{14}C-HMX amended soil.

9.3.3. <u>Uptake of non-labeled HMX in plants</u>

Study was conducted to determine uptake of HMX by ryegrass in SSL2007d soil using nominal HMX exposure concentrations 10, 30, 100, and 1000 mg/kg. At each time period (14, 21, 28, and 35 d), BCF were calculated by dividing the concentration of HMX in tissue by the measured concentration of HMX in the soil. Results showed that HMX accumulated mostly in shoots. The BCF values 27, 14, 3.9, and 0.3, were determined for 10, 30, 100, and 1000 mg/kg nominal soil HMX concentrations, respectively, based on HMX concentrations in ryegrass shoots after 35 d (Table 89). These BCF values are much lower than those observed previously for RDX at similar soil concentrations, thus suggesting a lesser accumulation on HMX in ryegrass compared to RDX.

Table 89. Summary of BCF calculated for plants exposed to HMX amended soil.

Days	BCF (SHOOTS)				BCF (ROOTS)			
	10 mg/kg	30 mg/kg	100 mg/kg	1000 mg/kg	Roots-10	Roots-30	Roots-100	Roots-1000
14	5.6	2.0	0.8	0.1	1.8	0.7	0.5	0.6
21	13.3	7.0	2.0	0.2	1.4	0.9	0.5	0.3
28	21.1	10.4	2.9	0.2	1.5	1.5	0.5	0.4
35	27.1	14.1	3.9	0.3	2.0	1.1	0.7	0.4

Table notes: BCF was calculated as the ratio of tissue to soil concentration at the respective exposure period.

9.3.4. Uptake of ^{14}C-HMX in plants

Analyses of shoots and roots from ^{14}C-HMX experiments, showed that radioactivity was mostly present in shoots (Figure 82). The amount of total radioactivity (including bound and unbound compounds) found in roots remains the same over the 35 d exposure period.

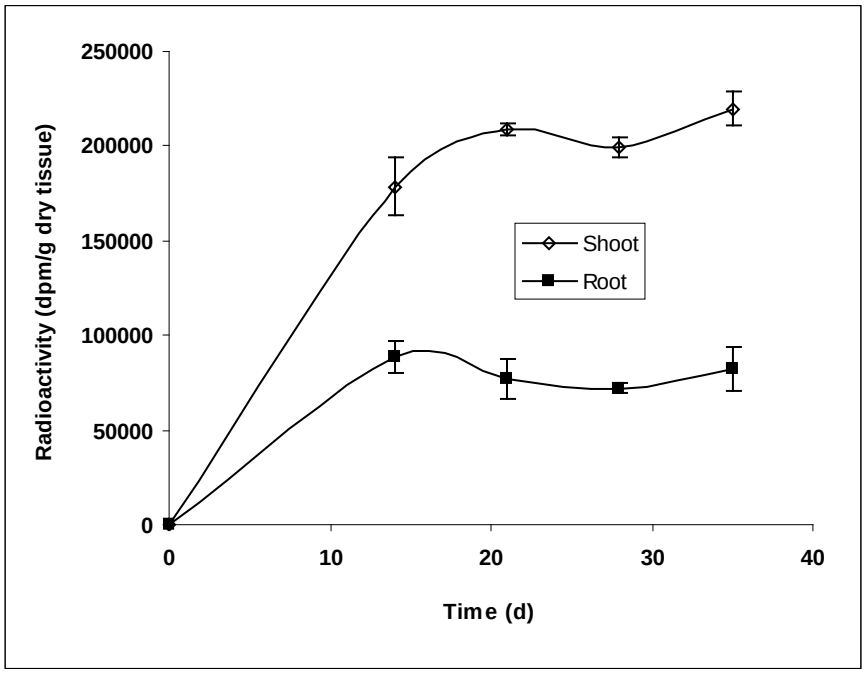


Figure 82. Radioactivity measured in plant tissues following exposure to ^{14}C-HMX amended soil for up to 35 d.

9.4. Bioaccumulation of 2,4-DNT

9.4.1. Uptake of 2,4-DNT in earthworms

The SSL2007d soil was amended with 2,4-DNT to prepare nominal target concentrations of 10, 20, and 50 mg/kg. These concentrations were selected based on survival experiments, and were not lethal to earthworms. Soil extracts showed similar trends in the presence or absence of earthworms. Results showed that 2,4-DNT decreased in soil as the exposure time increased (Figure 83). In addition, the two metabolites 2A-4-NT and 4A-2-NT were present. In soil extracts, total metabolites increased to the maximum values of 0.6, 1.6, and 6.3 mg/kg at 3 d in the 10, 20, and 50 mg/kg soil treatments, respectively. The amount of these metabolites in soil decreased after the 3–d exposure period (Figure 83).

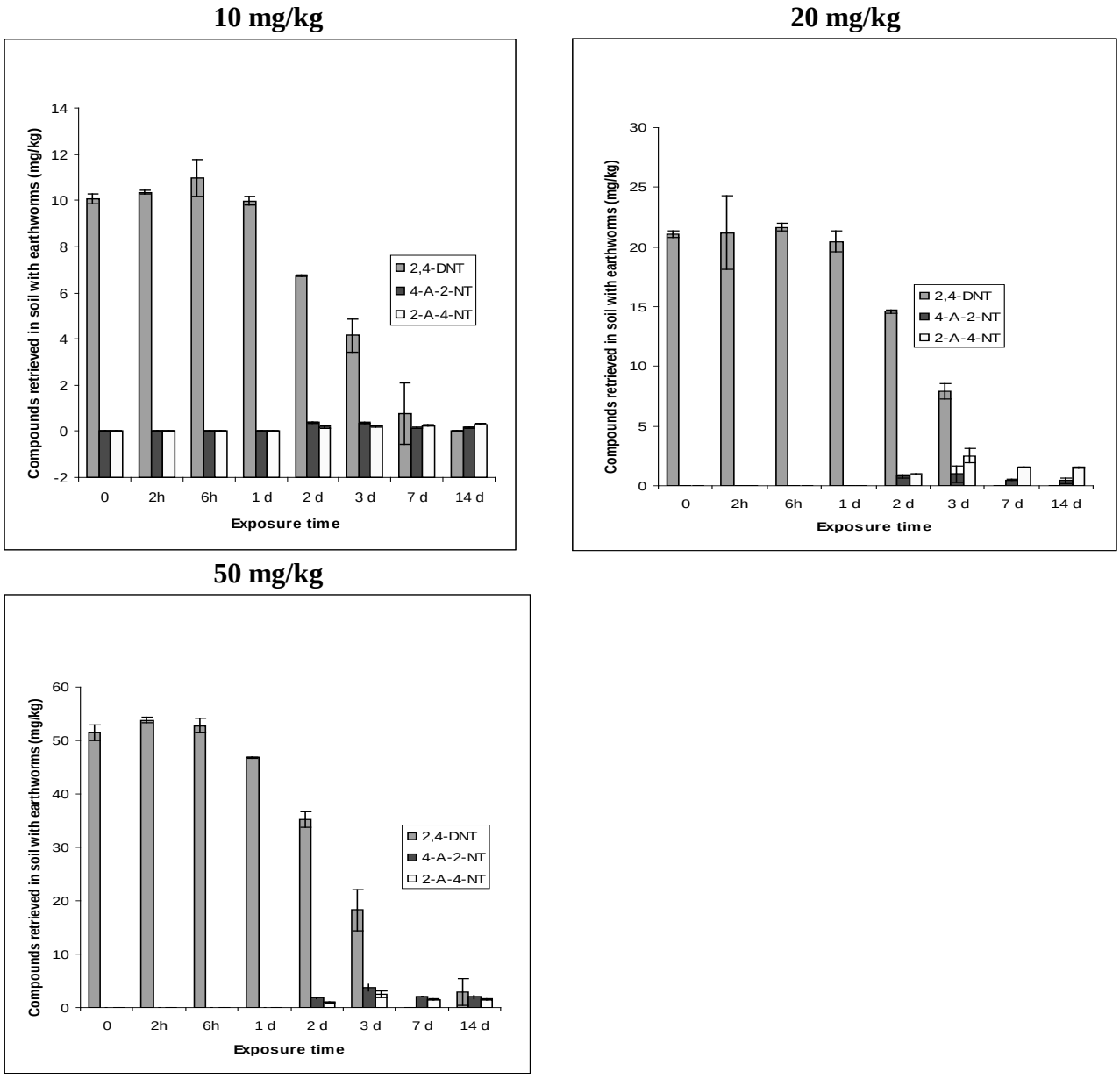
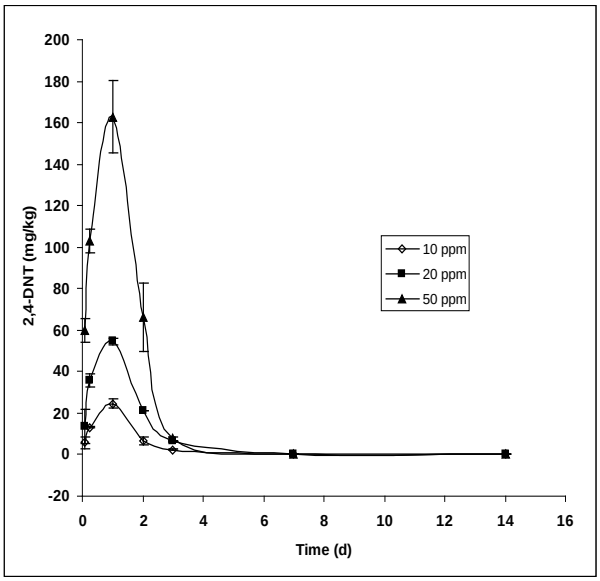
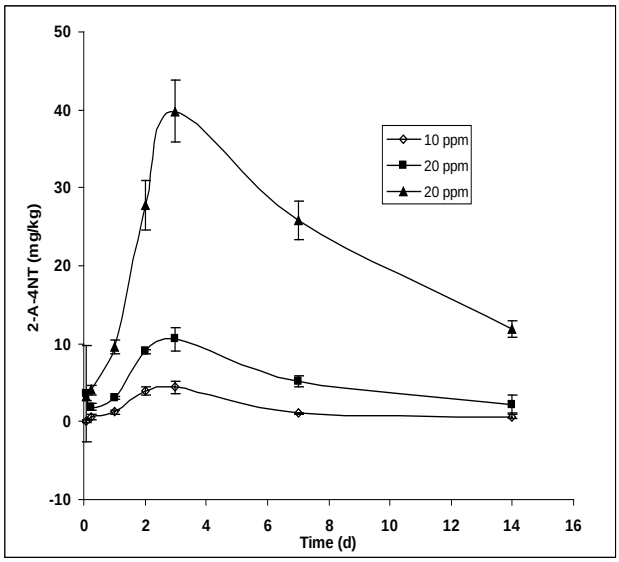


Figure 83. Concentrations of 2,4-DNT and its metabolites in soil treatments containing earthworms.





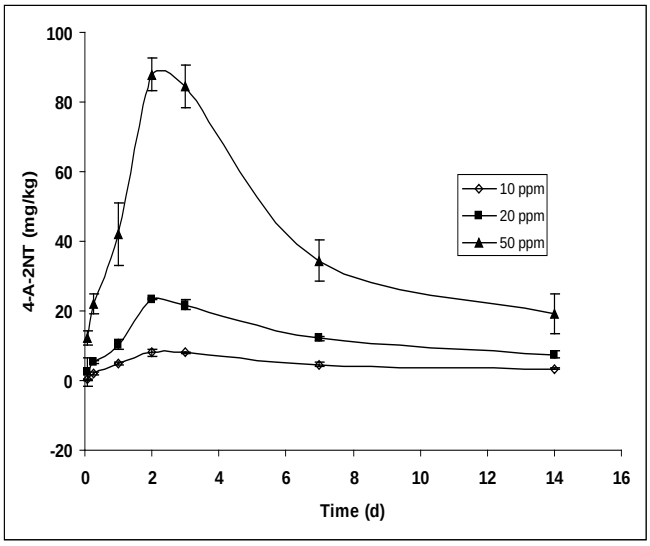


Figure 84. Compounds recovered in earthworms following exposure to 2,4-DNT amended soil.

Concentrations of 2,4-DNT increased in the earthworm tissues after 1 d of exposure, and then decreased to non-detectable levels after 7-d and 14-d exposure (Figure 84). The maximum amount of 2,4-DNT (163 mg/kg dry tissue) was extracted from the earthworms exposed for 1 d in soil amended at 50 mg 2,4-DNT/kg dry soil. Both 2A-4NT and 4A-2NT were found in all earthworms exposed in 2,4-DNT-amended treatments until the end of the assay. The amount of 2A-4NT was always lower than the amount of 4A-2NT found under the same soil treatment. The maximum amount of metabolites (40 and 84 mg/kg dry tissue for 2A-4NT and 4A-2NT, respectively) were found in earthworms exposed to 50 mg/kg for 3 d. These data indicate that the 2,4-DNT metabolites in earthworms could either originate from the soil or result from transformation of the parent compound by the earthworms. This study suggests that 2,4-DNT was metabolized and did not accumulate in earthworm tissues to any great extent compared with soil concentrations. Similar results were found in our studies with TNT.

9.4.2. Uptake of 2,4-DNT in plants

A study was conducted to determine the uptake of 2,4-DNT by ryegrass in SSL2007d soil using nominal concentrations of 1, 3, 5, and 10 mg/kg. Results indicate that concentration of 2,4-DNT decreased in all amended soils as the exposure time increased, including a 10% decrease in all 2,4-DNT concentration treatments within 24 h from initial hydration of freshly-amended soils (48 h from amending dry soils). No 2A-4NT or 4A-2NT (metabolites of 2,4-DNT) were detected in soil amended with 2,4-DNT concentration 1 mg/kg after any of the exposure periods. However, these metabolites were present in 2,4-DNT treatment concentrations greater than, and including 3 mg/kg after the 14-d and longer exposure periods (Tables 90 and 91).

Table 90. 2,4-DNT and its metabolites recovered from soil in the plant accumulation microcosm without plants added.

		Nominal concentration in soil (mg/kg)			
Expose time	Chemical	1	3	5	10
24 h	2,4-DNT	1.4 (0.03)	2.6 (0.1)	4.9 (0.2)	9.0 (0.3)
48 h	2,4-DNT	1.2 (0.1)	2.4 (0.1)	4.4 (0.02)	8.0 (0.2)
14 d	2,4-DNT	0.36	0.64	1.2	1.9
	2A-4NT	ND	ND	0.13	0.28
	4A-2NT	ND	ND	0.18	0.31
21 d	2,4-DNT	0.36	0.58	1.05	1.77
	2A-4NT	ND	ND	0.11	0.15
	4A-2NT	ND	ND	0.11	0.23
28 d	2,4-DNT	0.35	0.54	0.95	1.53
	2A-4NT	ND	ND	ND	0.15
	4A-2NT	ND	ND	ND	0.14
35 d	2,4-DNT	0.36	0.6	0.99	1.4
	2A-4NT	ND	ND	ND	0.12
	4A-2NT	ND	ND	ND	0.13

Table notes: Data are expressed as mean with standard deviation in brackets (n=3). No 2,4-DNT metabolites were detected in soil samples after 24 and 48 h hydration. ND: not detected (detection limit = 0.10 mg/kg).

Table 91. 2,4-DNT and its metabolites recovered from soil in the plant accumulation microcosm with plants added.

		Nominal concentration in soil (mg/kg)			
Expose time	Chemical	1	3	5	10
14 d	2,4-DNT	0.37 (0.01)	0.66 (0.04)	1.20 (0.06)	2.10 (0.01)
	2A-4NT	ND	ND	0.13 (0.02)	0.26 (0.03)
	4A-2NT	ND	ND	0.15 (0.02)	0.26 (0.03)
21 d	2,4-DNT	0.39 (0.04)	0.60 (0.04)	1.03 (0.06)	1.73 (0.05)
	2A-4NT	ND	ND	ND	0.18 (0.04)
	4A-2NT	ND	ND	ND	0.15 (0.03)
28 d	2,4-DNT	0.30 (0.01)	0.52 (0.03)	1.01 (0.06)	1.50 (0.08)
	2A-4NT	ND	ND	ND	0.14 (0.02)
	4A-2NT	ND	ND	ND	0.10 (0.02)
35 d	2,4-DNT	0.38 (0.04)	0.57 (0.02)	1.03 (0.04)	1.64 (0.12)
	2A-4NT	ND	ND	ND	0.10 (0.09)
	4A-2NT	ND	ND	ND	0.13 (0.00)

Table notes: Data are expressed as mean with standard deviation in brackets (n=3). ND: not detected (detection limit = 0.10 mg/kg).

The parent compound 2,4-DNT was found in ryegrass shoots following 14 d of plant exposure in soil to all 2,4-DNT concentrations, and after 21 d in the 3 and 5 mg/kg treatments. Concentrations of 2,4-DNT metabolites were below analytical quantification limit (AQL) for tissue of 0.7 mg/kg in shoots of ryegrass after 14 and 21 d of exposure. No 2,4-DNT or metabolites were found in the shoots above AQL after 28 and 35 d of exposure in any treatment concentrations. Concentrations of 2,4-DNT in the roots varied during the 35-d study. It was present in roots in all treatment concentrations after 14 d, in 5 and 10 mg/kg treatments after 21 and 28 d, and only in the greatest treatment concentration of 10 mg/kg after 35 d of exposure. Both metabolites of 2,4-DNT were consistently found in the roots throughout the 35-d study in the greatest treatment concentration of 10 mg/kg. Additionally, 2A-4NT was found in the roots in 1, 3, and 10 mg/kg treatments after 14 d (Tables 92 and 93).

Table 92. Compounds recovered from shoots of ryegrass following exposure to 2,4-DNT amended soil.

Nominal 2,4-DNT concentration in the amended soil (mg/kg)	Compounds measured in shoots after 14 d exposure in amended soil (mg/kg)			Compounds measured in shoots after 21 d exposure in amended soil (mg/kg)		
	2,4-DNT	2A-4NT	4A-2NT	2,4-DNT	2A-4NT	4A-2NT
1	2.8 (0.5)	ND	ND	ND	ND	ND
3	2.0 (0.2)	ND	ND	1.2 (0.5)	ND	ND
5	2.8 (0.4)	ND	ND	1.3 (0.7)	ND	ND
10	1.4 (0.1)	ND	ND	ND	ND	ND

Table notes: Data are expressed as mean with standard deviation in brackets (n=3). ND: not detected (detection limit = 0.7 mg/kg). No 2,4-DNT or metabolites were detected in shoots samples after 28 and 35 d.

Table 93. Compounds recovered from roots of ryegrass following exposure to 2,4-DNT amended soil.

		Nominal concentration in soil (mg/kg)			
Expose time	Chemical	1	3	5	10
14 d	2,4-DNT	2.7 (4.6)	1.4 (1.2)	1.9 (1.9)	2.8 (1.1)
	2A-4NT	7.1 (12.3)	2.3 (4.0)	ND	2.1 (0.3)
	4A-2NT	ND	ND	ND	1.3 (1.1)
21 d	2,4-DNT	ND	ND	3.2 (5.6)	3.2 (1.8)
	2A-4NT	ND	ND	ND	2.7 (0.9)
	4A-2NT	ND	ND	ND	3.9 (1.2
28 d	2,4-DNT	ND	ND	1.0 (1.8)	1.5 (0.3)
	2A-4NT	ND	ND	ND	2.2 (1.6)
	4A-2NT	ND	ND	ND	1.0 (0.7)
35 d	2,4-DNT	ND	ND	ND	1.2 (2.0)
	2A-4NT	ND	ND	ND	2.3 (4.0)
	4A-2NT	ND	ND	ND	0.7 (1.2)

Tables notes: Data are expressed as mean with standard deviation in brackets (n=3). ND: Not detected (detection limit = 0.7 mg/kg). No 2,4-DNT or metabolites were detected in shoots samples after 28 and 35 d.

The BCF (tissue to soil ratio of measured 2,4-DNT concentrations) were determined for all treatment concentrations when possible. After the 14-d exposure; BCF values were inversely related to 2,4-DNT concentration in soil. Specifically, the shoot-based BCF values were 7.6, 3.0, 2.3, and 0.7 for the 1, 3, 5, and 10 mg/kg treatments, respectively, and the root-based BCF values were 7.3, 2.1, 1.6, and 1.4 for the 10, 5, 3, and 1 mg/kg treatments, respectively (Table 94). The shoot-based BCF values of 2.0 and 1.3 were determined for the 3 and 5 mg/kg treatments, respectively, after 21 d, and no shoot-based BCF values could be determined for the remaining treatments after 21 d, or any other treatments after 28 or 35 d of exposure, which suggested a trend of decrease for the shoot-based BCF values over time. In contrast, the root-based BCF values ranging 0.7 to 1.8 were determined for the greatest 2,4-DNT treatment of 10 mg/kg throughout the 35-d study and for the second greatest treatment of 5 mg/kg after 21 and 28 d of exposure, where they were 3.1 and 1.0, respectively (Table 94). These results suggest that 2,4-DNT can be taken up by ryegrass from soil, and that 2,4-DNT can be transformed either in soil or in ryegrass roots into 2A-4NT and/or 4A-2NT. The absence of 2,4-DNT or its metabolites in plant tissues after the 28-d and greater exposure periods suggests that these compounds could be sequestered in ryegrass and therefore, could not be extracted. It is also possible that the compounds were mineralized completely, or transported to the roots, or eliminated from the plant via a variety of chemical transport mechanisms.

Table 94. Estimation of the bioconcentration factor (BCF) of 2,4-DNT for the different concentrations tested.

2,4-DNT nominal concentration in the amended soil	BCF calculated for different exposure time (mg tissue/kg soil)							
(mg/kg)	14 d Shoots	14 d Roots	21 d Shoots	21 d Roots	28 d Shoots	28 d Roots	35 d Shoots	35 d Roots
1	7.6	7.3	nd	nd	nd	nd	nd	nd
3	3.0	2.1	2.0	nd	nd	nd	nd	nd
5	2.3	1.6	1.3	3.1	nd	1.0	nd	nd
10	0.7	1.4	nd	1.8	nd	1.0	nd	0.7

Table notes: Data are expressed as ratio of tissue to soil concentrations; nd: not determined because compounds were below detection limits of 0.7 mg/kg for soil and 2.3 mg/kg for shoot or root tissue.

9.5. Bioaccumulation of NG

9.5.1. Uptake of NG in earthworms

Tests were conducted using NG amended into SSL2007d soil. Based on earthworm lethality test, four non-lethal concentrations (from 25 to 150 mg/kg) were selected for the bioaccumulation study. HPLC analyses of the soil extracts prior to testing showed the respective NG recoveries of 74 (19 mg/kg), 81 (41 mg/kg), 105 (105 mg/kg), and 96 (145 mg/kg) percent. No dinitroglycerin (DNG; a potential NG metabolite) was detected in soil samples prior to testing. The concentrations of NG and its metabolite (DNG) were monitored in the earthworms during the uptake phase. Transformation of NG in soil without earthworms was also measured. A decrease in NG concentration was observed at all concentrations tested up to and including 14 d of exposure. NG disappeared, or became non-extractable from soil, at 21 and 28 d of exposure. DNG was found in the extract from soil with and without earthworms up to and including 2 d of exposure. Very low or non-detectable levels of DNG were found in soil extracts after 2 d of experiment (Figure 85).

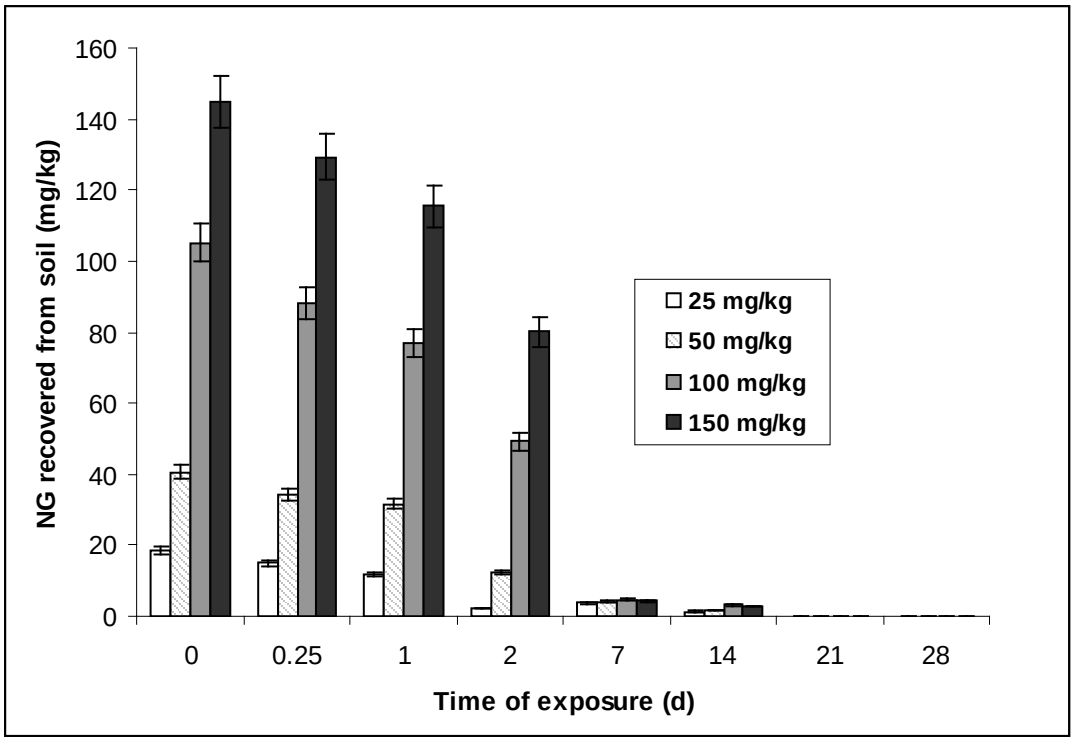


Figure 85. NG concentration decreased in soil in presence of earthworms.

Results also showed that NG did not accumulate in the earthworm tissue at any of the concentrations tested for up to and including 28 d of exposure. In contrast to the results of soil extractions no NG or DNG was found in earthworms at any of the concentrations and exposure period. After 14-d a peak was found in the UV-HPLC spectrum but was not consistent with the retention time of either NG or DNG.

9.5.2. Uptake of NG in plants

Study was conducted to determine the uptake of NG by ryegrass in SSL2007d soil using nominal NG concentrations of 10, 30, 50, and 75 mg/kg. Results in Table 95 indicate that NG decreased in soil as the exposure time increased. Analysis of soil samples indicates that NG metabolite (DNG) was present in the 14-d samples at NG treatment concentrations greater than, and including 30 mg/kg. The greatest amount of DNG in soil samples (14.8 mg/kg soil) was found in the NG treatment of 75 mg/kg after 14 d (Table 95). NG was not accumulated in either shoots or roots of ryegrass; however, DNG was found in shoots of ryegrass exposed to NG concentrations of 50 or 75 mg/kg (Table 95). The DNG was also found in roots of ryegrass exposed to NG concentrations of 30, 50, and 75 mg/kg.

Table 95. NG and DNG recovered from soil in the ryegrass plant microcosm.

	NG in soil (mg/kg)				DNG in soil (mg/kg)			
Time (d)	10	30	50	75	10	30	50	75
1	10.8 (8.5)	20.8 (0.4)	38.7 (0.4)	63.8 (1.4)	2.2 (1.1)	3.5 (0.1)	5.4 (0.3)	8.2 (2.1)
2	4.8 (0.1)	19.9 (0.6)	36.6 (1.2)	63.6 (2.1)	2.5 (0.4)	5.4 (0.2)	7.4 (0.7)	9.8 (0.6)
14	0.8 (0.2)	1.5 (0.1)	1.9 (0.3)	3.3 (0.6)	ND	1.1 (1.0)	5.5 (1.9)	14.8 (3.1)
21	0.9 (0.1)	1.0 (0.3)	1.7 (0.2)	2.0 (0.2)	ND	ND	ND	ND
28	ND	1.3 (0.2)	0.8 (0.3)	1.3 (0.2)	ND	0.2 (0.3)	0.7 (1.3)	ND
35	ND	ND	0.6 (0.5)	0.6 (0.1)	ND	ND	0.5 (0.9)	ND

Table notes: Data are expressed as mean with standard deviation in brackets (n=3). ND: Not detected (detection limit =0.1 mg/kg).

The greatest amount of DNG (1964 mg/kg tissue) was found in ryegrass roots in the NG treatment of 75 mg/kg after 21 d of exposure (Table 96 and Figure 86). These results suggest that NG can be transformed in ryegrass roots into DNG, which is subsequently translocated into the shoots.

Table 96. DNG measured in shoots and roots of ryegrass following exposure to NG amended soil.

Nominal concentration of	DNG recovered in shoots (mg/kg)				DNG recovered in roots (mg/kg)			
NG in soil (mg/kg)	14 d	21 d	28 d	35 d	14 d	21 d	28 d	35 d
10	ND	ND	ND	ND	ND	ND	ND	ND
30	ND	ND	ND	ND	295 (256)	469 (100)	111 (15)	13 (23)
50	194 (47)	ND	ND	ND	879 (73)	1262 (444)	493 (255)	170 (32)
75	596 (300)	486 (104)	69 (76)	212 (368)	1586 (525)	1964 (442)	744 (100)	208 (41)

Table notes: Data are expressed as mean with standard deviation in brackets (n=3). ND: not detected (detection limit =0.7 mg/kg).

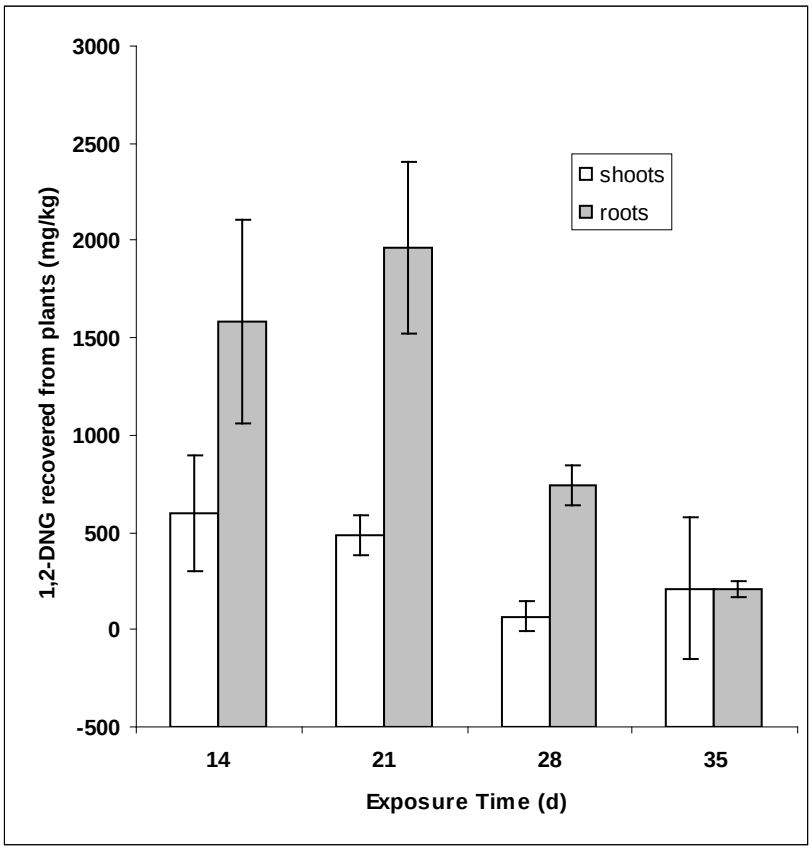


Figure 86. Presence of DNG in roots and shoots of ryegrass following exposure to NG amended soil.

10. Conclusions and Implications for Future Research/Implementation

Present studies were designed to develop critical benchmark data required for successful management of defense installations in a sustainable manner and for the knowledge-based decision making. Generating toxicity data to establish benchmarks that are appropriate for utilization in deriving the terrestrial plant-based and the soil invertebrate-based draft Eco-SSLs for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG was among the main objectives of the studies conducted in this project. Ecotoxicological testing in those studies was specifically designed to meet the criteria for Eco-SSL derivation outlined in the Eco-SSL Guideline (USEPA, 2005). Definitive studies using terrestrial plants and soil invertebrates exposures in upland aerobic sandy loam soils established new ecotoxicological data for 2,4-DNT, 2-ADNT, 4-ADNT, HMX, and NG effects on plants and soil invertebrates under conditions of very high relative bioavailability for organic chemicals in soil (as defined in USEPA, 2005). The draft Eco-SSL values detailed in this report were derived utilizing EC_{20} benchmark values for EM effects on plant growth or soil invertebrate reproduction, and these measurement endpoints were determined from standardized toxicity tests. The preference for growth (plant) and reproduction (soil invertebrate) benchmarks, and for low effect level (*i.e.*, EC_{20}), was justified to ensure that Eco-SSL values would be protective of populations of the majority of ecological receptors in soil, and provide confidence that EM concentrations posing an unacceptable risk are not screened out early in the ERA process (*i.e.*, SLERA).

Toxicity testing was also conducted with additional natural soil types to extend the range of soil physico-chemical characteristics that were hypothesized to affect the 2,4-DNT toxicity to plants and soil invertebrates. Soil-related differences were evident in phytotoxicity benchmarks, and in both acute (adult survival) and chronic (cocoon or juvenile production) toxicity benchmarks established in studies with earthworms or potworms exposed to 2,4-DNT weathered-and-aged in each of the natural soils tested in the present studies. The quantitative analyses of relationships among the acute or chronic toxicity benchmarks for 2,4-DNT and soil property measurements revealed that both clay and organic matter contents of the soil affected toxicity of 2,4-DNT to the earthworms and potworms. Strong correlations were also detected for several annelid (earthworms and potworms) toxicity endpoints and soil CEC (which depends upon both soil clay and soil organic matter content). Organic matter content of the soil was also strongly correlated with plant growth toxicity benchmarks for 2,4-DNT. No significant correlations were found among any toxicity benchmarks for 2,4-DNT and soil pH. These results identified soil organic matter and clay as the dominant properties mitigating 2,4-DNT toxicity for soil annelids, and organic matter as the soil constituent mitigating 2,4-DNT toxicity for plants.

Assessment and protection of the terrestrial environment at defense installations will be advanced by additionally applying scientifically-based ecotoxicological benchmarks for biologically-mediated processes in soil established in present studies. Soil microorganisms are critical to terrestrial biogeochemical cycles and as such are essential to sustaining the soil fertility in the terrestrial ecosystems. Consequently, assessment of the effects on soil microorganisms should be an integral part of testing requirements for the environmental risk assessment of chemicals in soil. Our results showed that soil contamination with 2,4-DNT, 2-ADNT, 4-ADNT, and NG can alter the rates of biologically-mediated processes in soil by either inhibiting or stimulating the soil microbial activity at the affected sites. Basal respiration and dehydrogenase

activity assays were the most robust among the soil functional tests used in the present studies and allowed us to establish toxicity data for each of the four EM investigated in this project. These assays can be recommended for inclusion into a battery of toxicity tests for assessing the effects of soil contamination with EM. Ecotoxicological benchmark values determined in the present studies for biologically-mediated processes in soil were generally comparable to those devoloped in plant and soil invertebrate toxicity tests. All toxicity benchmarks were developed using freshly collected soil, and were based on the analytically measured EM concentrations. These benchmarks will allow screening of site soil data to identify those EM contaminants that are of potential ecological concern, thus should be further considered in a BERA. These benchmarks for the soil microbial activity will provide a useful tool for the DoD installation managers to gauge the ecotoxicological impacts of the military operations that involve the use of explosives and propellants, thus ultimately promoting the sustainable use of testing and training ranges by today's and future Warfighters.

Another objective of the studies conducted in this report was to determine bioaccumulation potential of five compounds: RDX, HMX, TNT, 2,4-DNT, and NG in earthworms and plants. For both species, determining bioaccumulation or bioconcentration factors (BAF; BCF) for ecological risk assessment had limitations when dealing with different soil characteristics or amendments. These limitations include and are not limited to saturation of pore water at high concentration of the contaminant; time-dependent metabolism of nitroaromatics and NG, effect of contaminant on lipid content of the invertebrate. The general protocol was published using RDX as a model compound, and could also be used for other explosives as well as other soil invertebrates. A protocol for plant studies was also published with NG results and used ryegrass as model plant species.

These present studies have provided critical ecotoxicological benchmarks for ERA of the EMs investigated, plus provided valuable insites into soil processes that impact the respective EM toxicities and that also affect EM bioavailabilities in soil. Currently, USEPA Guidance (USEPA, 2005) specifies that such ecotoxicological benchmark data be utilized in the early stages of ERA (e.g., SLERA), yet another aspect of concern arises when moving to the BERA. EMs that contaminate testing and training ranges almost always occur as mixtures of compounds in the environment. Therefore, future research should include determinations of synergistic ecotoxicological affects of frequently utilized combinations of EMs. Additional research should also be undertaken to assess the effective biological accessability of such mixtures and resulting transformation products over time, since these may ultimately have the greatest long-term effects on the ecosytem and environment.

11. References

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12. Appendix

12.1. Presentations at conferences

2011

Kuperman, R.G., Dodard, S., Checkai, R.T., Minyard, M., Phillips, C.T., Simini, M., Rocheleau, S., Hawari, J., and Sunahara, G.I. 2011. Effects of energetic materials on soil biological activity processes in Sassafras sandy loam. Soil Ecology Society Biennial Conference, The University of British Columbia (Okanagan Campus), Kelowna, B.C. Canada, 24-27 May 2011 (published abstract).

Kuperman, R.G., Minyard, M., Checkai, R.T., Rocheleau, S., Dodard, S., Sarrazin, M., Paquet, L., Hawari, J., and Sunahara, G.I. 2011. Soil screening concentrations for energetic soil contaminants: Effects on soil microbial activity in Sassafras sandy loam. Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, 29 November -1 December, 2011 (published abstract).

Minyard, M., Kuperman, R.G., Checkai, R.T., Hawari, J., and Sunahara, G.I. 2011. Effects of dinitrotoluenes on carbon mineralization and microbial biomass in Sassafras sandy loam. Society of Environmental Toxicology and Chemistry 32nd Annual Meeting, Boston, MA. 13-17 November 2011 (published abstract).

Minyard, M., Kuperman, R., Checkai, R.T., Rocheleau, S., Hawari, J., and Sunahara, G.I. 2011. Effects of dinitrotoluenes and nitroglycerin on carbon mineralization and microbial biomass in Sassafras sandy loam soil. SETAC Annual meeting, Boston, MA, November 2011 (published abstract).

Sunahara, G.I., Kuperman, R.G., Checkai, R.T., Simini, M., Hawari J., Ampleman, G., and Thiboutot, S. 2011. Development of environmental tolerance values for defense sites contaminated with energetic materials. Presented at the NATO-RTO, AVT Symposium on Munition and Propellant Disposal and its Impact on the Environment (Edinburgh, UK, 17-20 October 2011).

2010

Checkai, R.T., Kuperman, R.G., Simini, M., Phillips, C.T., Rocheleau, S., Hawari, J., and Sunahara, G.I. 2010. Soil invertebrate and terrestrial plant based toxicity benchmarks: Bioavailability and ecological soil screening levels for energetic materials. Soil Science Society of America Annual Meeting, Long Beach, CA. 31 October - 3 November 2010 (published abstract).

Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T., Rocheleau, S., Sunahara, G.I., and Hawari, J. 2010. Can ecologically protective soil screening concentrations for energetic materials be predicted from the site-specific soil properties? Society of Environmental Toxicology and Chemistry 31st Annual Meeting, Portland, OR. 07-11 November 2010 (published abstract).

Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T., Rocheleau, S., Sunahara, G.I., and Hawari, J. 2010. Development of Ecological Soil Screening Levels (Eco-SSL) for nitrogen-based propellant materials using soil invertebrate and terrestrial plant toxicity benchmarks. Joint Army-Navy-NASA-Air Force (JANNAF) Meeting, Orlando, FL, 06-10 December 2010 (published abstract).

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2009

Dodard, S., Kuperman, R.G., Sarrazin, M., Savard, K., Joly, M., Hawari, J., Ampleman, G., Thiboutot, S., Simini, M., Checkai, R.T., Robidoux, P.I., and Sunahara, G.I., 2009. Uptake of 2,4,6-trinitrotoluene, 2,4-dinitrotoluene, and nitroglycerin by soil invertebrates and plants in a sandy loam soil. Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, 1-3 December, 2009 (published abstract).

Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T., Rocheleau, S., Sunahara, G.I., and Hawari, J. 2009. Benchmark toxicity data for energetic materials for developing the soil invertebrate-based Ecological Soil Screening Levels (Eco-SSL). Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, 1-3 December, 2009 (published abstract).

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2008

Kuperman, R.G., Checkai, R.T., Römbke, J., Stephenson, G.L., and Sousa, J.P. 2008. Ecotoxicological assessment of contaminated land. 15th International Colloquium on Soil Zoology, Curitiba, PR Brasil 25-29 August 2008 (published abstract).

Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T., Sunahara, G.I., Hawari, J., and Rocheleau, S. 2008. Toxicity of nitroglycerin and aminodinitrotoluenes to potworm *Enchytraeus crypticus* in a sandy loam soil. The 2008 Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, 2-4 December, 2008 (published abstract).

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Phillips, C.T., Checkai, R.T., Kuperman, R.G., and Simini, M. 2008. Ecotoxicity of 2-ADNT and NG: Effects on *Folsomia candida* of 2-ADNT and NG weathered-and-aged in a natural soil. The 2008 Partners in Environmental Technology Technical Symposium & Workshop, Washington, DC, 2-4 December, 2008 (published abstract).

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2007

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12.2. Supporting data: List of scientific and technical publications generated by this project

12.2.1. Book

Sunahara, G.I., Lotufo, G., Kuperman, R.G., and Hawari, J. (Eds). *Ecotoxicology of Explosives.* CRC Press, ISBN: 978-0-8493-2839-8, 336 Pages, June 2009.

12.2.2. Book chapters

2011

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2009

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12.2.3. Peer-reviewed publications

2011

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2010

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Rocheleau, S., Kuperman, R.G., Simini, M., Hawari, J., Checkai, R.T., Thiboutot, S., Ampleman, G., and Sunahara, G.I. 2010. Toxicity of 2,4-dinitrotoluene to terrestrial plants in natural soil. *Sci. Tot. Environ.* 408: 3193-3199.

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2008

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*12.2.4. **Technical publications:***

2012

Checkai, R.T., Kuperman, R.G., Simini, M., and Phillips, C.T. (2012). Derivation of draft ecological soil screening levels for TNT and RDX utilizing terrestrial plant and soil invertebrate toxicity benchmarks. Technical Report No. ECBC-TR-963, U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD.

Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T., Kolakowski, J.E., and Kurnas, C.W. (2012). Acute and chronic toxicities of TNT and RDX to the enchytraeid worm, *Enchytraeus crypticus*, in natural soils. Technical Report No. ECBC-TR-981, U.S. Army Edgewood Chemical Biological Center: Aberdeen Proving Ground, MD.

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2007

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Sunahara, G.I., Kuperman, R.G., Rocheleau, S., Dodard, S., Paquet, L., Sarrazin, M., Savard, K., Checkai, R.T., Simini, M., Phillips, C.T., Thibouthot, S., Ampleman, G., and Hawari, J., 2007. Development of toxicity benchmarks and bioaccumulation data for N-based organic explosives for terrestrial plants and soil invertebrates. Strategic Environmental

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2006

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Kuperman, R.G., Checkai, R.T., Simini, M., Phillips, C.T., Kolakowski, J.E., Kurnas, C.W. 2006. Effect of simulated weathering and aging of TNT in amended sandy loam soil on toxicity to enchytraeid worm *Enchytraeus crypticus*. Technical Report No. ECBC-TR-484. U.S. Army Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD, May 2006.

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2005

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12.3. Other supporting materials: Awards

Bardai, G., Hales, B.F., and Sunahara, G.I. Role of nitrite in glyceryl trinitrate (GTN) induced microphthalmia in quail embryos. *The Teratology Society* 49th Annual Meeting held in Rio Grande, Puerto Rico from June 26 to July 1, 2009. This oral presentation won 1st prize in the James G. Wilson Presentation Award Competition.

Members of the SERDP ER1416 project research team were co-recipients of the *2010 TTCP International Scientific Achievement Award.* TTCP stands for The Technical Cooperation Program of International Defense Departments and Ministries of Canada, UK, Australia, USA, and NZ. See SERDP Newsletter 09/30/2011 - http://www.serdp.org/News-and-Events/News-Announcements/Program-News/Award-winning-research-on-ecotoxicology-of-energetics-presented-at-NATO-conference/%28language%29/eng-US

12.4. Published manuscripts

Publications indicated in Sections 9.1.1 and 9.1.2.

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ACCUMULATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE BY THE EARTHWORM *EISENIA ANDREI* IN A SANDY LOAM SOIL

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Abstract—The heterocyclic polynitramine hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a highly energetic compound found as a soil contaminant at some defense installations. Although RDX is not lethal to soil invertebrates at concentrations up to 10,000 mg/kg, it decreases earthworm cocoon formation and juvenile production at environmentally relevant concentrations found at contaminated sites. Very little is known about the uptake of RDX in earthworms and the potential risks for food-chain transfer of RDX in the environment. Toxicokinetic studies were conducted to quantify the bioaccumulation factors (BAFs) using adult earthworms (*Eisenia andrei*) exposed for up to 14 d to sublethal concentrations of nonlabeled RDX or [^{14}C]RDX in a Sassafras sandy loam soil. High-performance liquid chromatography of acetonitrile extracts of tissue and soil samples indicated that nonlabeled RDX can be accumulated by the earthworm in a concentration- and time-dependent manner. The BAF, expressed as the earthworm tissue to soil concentration ratio, decreased from 6.7 to 0.1 when the nominal soil RDX concentrations were increased from 1 to 10,000 mg/kg. Tissue concentrations were comparable in earthworms exposed to nonlabeled RDX or [^{14}C]RDX. The RDX bioaccumulation also was estimated using the kinetically derived model (BAF_K), based on the ratio of the uptake to elimination rate constants. The established BAF_K of 3.6 for [^{14}C]RDX uptake was consistent with the results for nonlabeled RDX. Radioactivity also was present in the tissue residues of [^{14}C]RDX-exposed earthworms following acetonitrile extraction, suggesting the formation of nonextractable [^{14}C]RDX metabolites associated with tissue macromolecules. These findings demonstrated a net accumulation of RDX in the earthworm and the potential for food-chain transfer of RDX to higher-trophic-level receptors.

Keywords—Earthworm Hexahydro-1,3,5-trinitro-1,3,5-triazine Explosives Bioaccumulation Soil

INTRODUCTION

The polynitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) can adversely affect soil invertebrates at environmentally relevant concentrations found at defense installations [1–4]. Earthworm exposure to RDX adversely affected reproduction, based on the median effective concentrations (EC50s) of 3.7 and 5.0 mg/kg for cocoon and juvenile production, respectively, but did not affect the survival of adult earthworms at concentrations up to and including 756 mg/kg in a 56-d test [5–8]. Sublethal effects of RDX in earthworms included neurotoxicity at 0.21 μg/cm^2 in a 14-d filter-paper study [9].

The toxicity of RDX or its nitroso-metabolites also was demonstrated for different vertebrate species [8,10]. In fact, RDX occasionally has been used as a rat poison [11]. Subchronic toxicity studies using Fischer 344 rats receiving daily doses of 100 mg RDX/kg body weight for 90 d showed that RDX can cause lethality, specific organ toxicities, and neurological effects [12]. Acute oral toxicity studies showed that the RDX metabolite hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX) caused 100% lethality at 400 mg/kg in gavaged Sprague–Dawley rats [13]. Convulsions also were observed in MNX-treated animals. Acute toxicity studies showed that oral doses of RDX greater than 630 mg/kg were lethal to Northern Bobwhite [14]. Central nervous system and respiratory distresses also were observed in the RDX-exposed birds.

In humans, RDX is a possible carcinogen, and accidental exposure to RDX has led to central nervous system problems, nausea, and vomiting [11]. These toxicological data for RDX and its metabolites suggest that soil RDX concentrations found at defense installations [1,15] can pose health risks to humans and wildlife through food-chain transfer [10]. Consequently, a better understanding of RDX accumulation in soil invertebrates, including earthworms that are key components of soil trophic webs, requires further study.

Bioaccumulation of environmentally persistent chemicals in animals involves several interacting physiological processes that govern the uptake of a contaminant by an organism following dermal absorption or ingestion, as described elsewhere [16–18]. The accumulation of a chemical in an organism often is conveyed through a bioaccumulation factor (BAF), which can be expressed as the steady state–based distribution coefficient of the tissue to soil concentrations of the test compound [19–21]. Sunahara et al. [22] determined the BAFs for RDX in laboratory microcosm studies with the earthworm *Eisenia andrei* and reported that the BAFs decreased from 13 to 2.9 as nominal RDX concentrations in a Sassafras sandy loam (SSL) soil increased from 10 to 100 mg/kg dry soil. Statistical analysis of these data revealed a log-linear relationship between RDX concentrations in soil and in earthworms [23]. Best et al. [24] determined an average

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tissue-to-soil RDX concentration ratio of 1 in a 28-d study with *E. fetida* exposed to a field-collected soil containing RDX and other contaminants, including octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX) and 1,3-dinitrobenzene. Those authors did not comment on whether the presence of the latter co-contaminants had an effect on RDX accumulation in the earthworm. Discrepancies in the published BAFs for RDX can be attributed to differences in the experimental designs used in the latter studies, including the methods used to assess bioaccumulation, the earthworm species, soil properties, RDX concentrations, duration of exposures, presence of co-contaminants in field-collected soils, and the formation of RDX transformation products in soil or in the organism.

In addition to the steady state–based tissue-to-soil distribution coefficient model described above, the BAF can be estimated using the kinetic approach (BAF_K) [17,25,26]. For example, the BAF_K can be expressed as the ratio of the uptake and elimination rate constants for a test compound using a first-order, single-compartment model. This method has been used to estimate the BAF_K for RDX in aquatic vertebrate and benthic oligochaetes [27,28].

In the present study, the hypothesis that RDX can accumulate in soil invertebrates, such as earthworms, from RDX-amended soil was tested. The objective was to determine and contrast the BAFs for RDX in soil using two approaches: the steady state–based distribution coefficient model (ratio of tissue to soil RDX concentrations) using earthworms exposed to nonlabeled RDX, and the kinetic approach (BAF_K) using earthworms exposed to [^{14}C]RDX. Mass-balance estimation using [^{14}C]RDX was included to determine the fate of RDX and its metabolites in the soil and tissue.

MATERIALS AND METHODS

Chemicals and reagents

Nonlabeled RDX (Chemical Abstracts Service no. 121-82-4; 99.9% purity, with <0.1% MNX) and [^{14}C]RDX (specific activity, 54.4–62.1 μCi/mmol) were obtained from the Defense Research Development Canada–Valcartier. Authentic reference standards, including RDX, MNX, hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) were purchased from AccuStandard. All other chemicals were of reagent grade and were obtained from commercial suppliers. Acetone and acetonitrile (high-performance liquid chromatography [HPLC] grade) were obtained from Caledon Laboratories. The American Society for Testing and Materials (ASTM) type I water [29] was obtained using a Zenopure Mega-90 water purification system (Zenon Environmental) and was used throughout the present study. Glassware was washed with phosphate-free detergent and rinsed with acetone, nitric acid (10%, v/v), and deionized water.

Culturing and handling of earthworms

Adult earthworms (*E. andrei*) obtained from Carolina Biological Supply were used to establish the initial laboratory cultures. Animals were maintained in earthworm bedding (Magic Products) supplemented with dry cereal (Magic Worm Food; Magic Products). The incubator was kept under a 16:8-h light:dark photoperiod with a light intensity of 800 ± 400 lux (mean ± standard deviation), temperature of 20 ± 1°C, and relative humidity of 70 to 80%. Clitellated earthworms weighing from 425 to 690 mg were used in the present study.

Soil handling and amendments

A natural soil (SSL; fine-loamy, siliceous, mesic semiactive, Typic Hapludult) [30] collected from a grassland field on the property of the U.S. Army Aberdeen Proving Ground (MD, USA) was used in the present study. The physical and chemical characteristics of the SSL soil (11% clay, 1.2% organic carbon, 71% sand, and 18% silt; pH 5.5) were expected to support high relative bioavailability of RDX according to the ecological soil screening level (Eco-SSL) criteria [31] (http://www.epa.gov/superfund/health/exposure/bioavailability/guidance.htm). The SSL soil was air-dried, sieved on a 2-mm mesh screen, and weighed separately to prepare each treatment batch in a glass dish. Soil was spread to a thickness of approximately 2.5 to 4 cm. Nonlabeled RDX was dissolved in acetone and added to each soil batch to prepare target concentrations of 1, 10, 100, 1,000, 3,000, or 10,000 mg/kg. The selection of RDX concentrations in soil was based on published results of the earthworm toxicity tests [6,7]. Acetone solutions of RDX were poured evenly across the soil surface, ensuring that the volume of solution added did not exceed 15% (v/w) of the dry soil mass. The greatest concentration (10,000 mg/kg) was prepared in several steps using a stock solution of 40 g/L, each time not exceeding 15% of soil weight. Acetone was allowed to volatilize for 2 h between the steps [3,32]. The amended soil batches were kept in a darkened chemical hood for a minimum of 48 h to allow acetone evaporation [33]. Each soil treatment batch was transferred into high-density polyethylene containers coated with a Teflon®-like fluorocarbon and covered with aluminum foil to prevent photolysis of RDX. The soil batches were mixed overnight (18 ± 2 h) using a three-dimensional rotary soil mixer. Three replicates from each dry soil batch were hydrated individually to 70 to 75% of the SSL water-holding capacity (21% water based on the dry SSL soil mass) for 3 h before the beginning of the experiment.

Uptake of nonlabeled RDX by earthworms in amended soil

The RDX uptake experiments were performed with SSL soil using *E. andrei* according to the ASTM standard guide for soil bioaccumulation studies [34] with some modifications. Instead of plastic containers (as recommended in the 2004 ASTM guidelines [34]), glass containers were used to avoid adsorption of RDX to the container walls. Earthworms were acclimated for 24 h in nonamended SSL soil before the experiment. One earthworm per 10 g dry weight of soil was placed into each replicate test unit (250-ml glass jar) containing 60 or 100 g dry weight of soil.

Two grams of dry cereal were added to each test unit. Each test unit was then covered by a perforated lid to control soil moisture. Earthworm wet weights were recorded at the start of the experiment. Measurements of RDX in tissue and soil samples were taken by a time-series sampling in which earthworms were sampled destructively after 0.25, 1, 2, 4, 7, and 14 d of exposure. On each sampling day, the earthworms were collected, counted, rinsed with ASTM type I water, and depurated for 24 h on a moistened filter paper. Then, earthworms were rinsed, blotted dry, placed into glass tubes, and immediately frozen at −80°C. Soil samples from each replicate container were homogenized and stored at −20°C until processed for HPLC analysis. Chemical analyses were conducted on triplicate samples of soil or tissue that were collected from each treatment group on the designated sampling days.

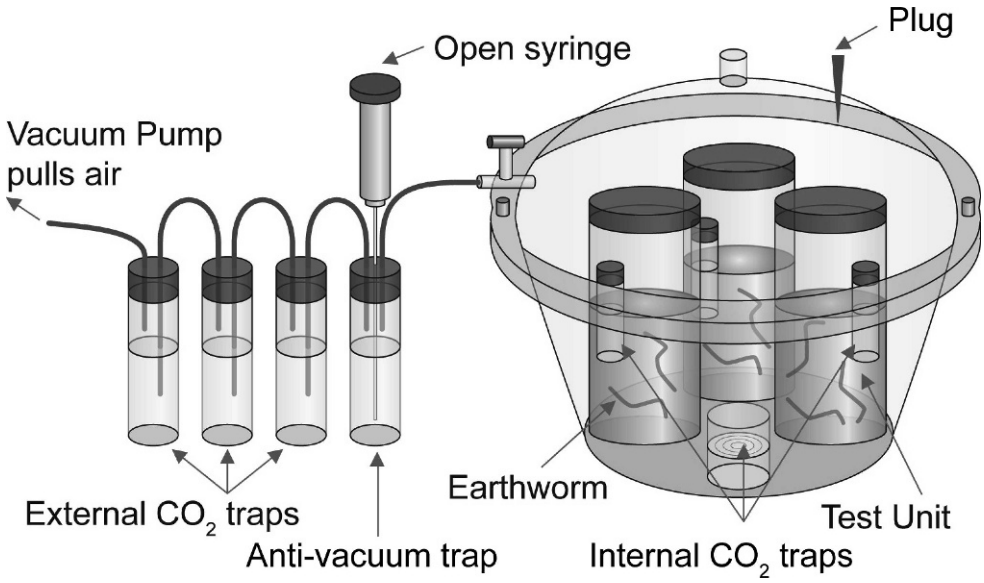

Fig. 1. Earthworm accumulation microcosm (EAM), a closed system. Adult *Eisenia andrei* were exposed to SSL soil amended with [^{14}C]hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). Internal and external $^{14}CO_2$ traps contained 0.5 N KOH. The EAM used in the present study was a modification of an earlier design described by Sunahara et al. [22] and by Kuperman et al. (http://www.serdp.org/research/upload/cu-1221-fr-01.pdf).

Chemical analyses of nonlabeled RDX in soil and earthworm samples

A modified U.S. Environmental Protection Agency Method 8330B [35] was used to extract and quantify the concentrations of RDX or its degradation products in the soil samples, using HPLC as described by Dodard et al. [33]. Triplicate soil samples (2 g each) were collected from each test unit, after which 10 ml of acetonitrile were added and the mixture vortexed for 1 min. Samples were sonicated for 18 h and then diluted (1:1, v/v) with 5 g/L of $CaCl_2$. The clear fraction was filtered through a 0.45-µm Millex™ HV cartridge (Millipore) before HPLC analyses. The limit of detection for RDX, MNX, and TNX in liquid samples was approximately 50 µg/L. Precision was 95% or greater (standard deviation, <2%). The laboratory detection limit for RDX, MNX, and TNX in SSL soil was 0.25 mg/kg, based on a signal-to-noise ratio of 10.

Tissue extracts of the earthworms exposed to nonlabeled RDX or of those in the control treatments (no RDX added) were prepared as described by Renoux et al. [5]. For each replicate, all earthworms were lyophilized and crushed using a mortar and pestle to obtain dry material for analysis. Two milliliters of ASTM type I water (4°C) were added to each tissue sample (0.48 ± 0.08 g), followed by vortexing for 10 s. Next, 5 ml of acetonitrile were added to the suspension, which then was vortexed for an additional 60 s. All samples were sonicated (Branson Model 3200) for 18 ± 2 h at 20°C and centrifuged (12,000 *g*) for 10 min at 4°C using a Sorval Super T21 centrifuge (Global Medical Instrumentation). A 3.5-ml aliquot of each supernatant was taken and mixed with 1.5 ml of $CaCl_2$ (16 g/L) and placed at 4°C for 2 h to precipitate the fine particles. The supernatant was filtered through a 0.45-µm Millex™ HV cartridge before HPLC analysis. The detection limit for nonlabeled RDX in the earthworm was 5 mg/kg dry tissue. Nitramine concentrations in earthworm tissue and soil were expressed as mg/kg dry tissue and mg/kg dry soil, respectively.

Uptake and elimination of [^{14}C]RDX by earthworms

The kinetics-based bioaccumulation test consisted of two phases (i.e., a period of uptake, followed by a period of elimination) as described by others [26,36]. The RDX uptake kinetics in earthworms were quantified by a time-series sampling in which earthworms were sampled destructively after 0.25, 1, 2, 3, 7, 9, and 14 d of exposure to 100 mg [^{14}C]RDX/kg dry SSL soil. Earthworms were depurated for 24 h on a moistened filter paper as described for the nonlabeled RDX studies. Based on the nonlabeled RDX exposure studies described above, this exposure concentration was not lethal to earthworms. Preparation of the [^{14}C]RDX-amended SSL soil followed the same procedure as described for the nonlabeled RDX soil studies.

At the start of the experiment, test units were placed into separate earthworm accumulation microcosms (EAMs) (Fig. 1) constructed from clear polycarbonate vacuum desiccators (inner diameter, 23 cm). Each EAM consisted of a maximum of six test units containing the earthworms and the [^{14}C]RDX-amended SSL soil. Control treatments were placed into separate EAMs. The EAM was made air-tight using two metal rings and associated polytetrafluoroethylene-rubber O-rings, fastened by bolted nuts. A 3-mm access port on the top of each EAM allowed sampling of the internal EAM alkali trap for CO_2.

All CO_2 traps contained 0.5 M KOH. Two sets of CO_2 traps were used to collect the evolved $^{14}CO_2$ within the EAM (Fig. 1). The first set of traps consisted of 10-ml glass tubes placed into each separate test unit and a 20-ml beaker placed outside the test units but inside each EAM. The second set of traps consisted of four external, serially connected test tubes. The first external tube from the EAM contained water and acted as the antivacuum trap. The remaining external tubes contained 20 ml of KOH each and trapped the evolved $^{14}CO_2$. The fourth tube also contained an outlet to flush air three times each week using a vacuum pump. Based on the results of preliminary studies, total air flush of the EAM was completed within 4 h.

On the designated sampling day, each external trap was sampled (1 ml) and mixed with ASTM type I water (1 ml) after each air flush. A 1-ml aliquot also was taken from each internal CO_2 trap. Scintillation counting fluid (18 ml) was added to the samples, and radioactivity was determined using a Packard Tri-Carb™ 2100TR (Canberra) liquid scintillation counter (LSC).

To quantify the RDX elimination kinetics, the earthworms exposed to [^{14}C]RDX for 14 d were removed from the soil, rinsed with ASTM type I water, and then transferred (without depuration on filter paper) into test units, with each test unit having 60 g of nonamended SSL soil. Triplicate test units then were placed into separate EAMs for subsequent destructive sampling. The RDX elimination kinetics in earthworms were quantified by a time-series destructive sampling after 0.08, 0.25, 1, 2, 3, 7, and 14 d of exposure in nonamended SSL soil. Earthworms were depurated for 24 h on a moistened filter paper as described for the RDX uptake studies. The concentrations of [^{14}C]RDX and its [^{14}C]metabolites (including MNX or TNX) in the earthworms were determined using the LSC or ^{14}C-HPLC during the uptake and elimination phases of this bioaccumulation test. The total ^{14}C-activity (RDX and its metabolites) in the individual test soil or tissue samples was determined using a PerkinElmer Model 307 sample oxidizer and LSC.

The [^{14}C]RDX uptake studies also were done to quantitate the mass balance using the EAM setup (Fig. 1) and triplicate test units containing earthworms (n = 10 worms/unit and 3 units/EAM) exposed to 100 mg [^{14}C]RDX/kg SSL soil for up to 14 d before destructive sampling and radiochemical analyses. Data are expressed as the percentage recovery of radioactivity in soil or earthworm or the evolved $^{14}CO_2$ relative to the amount of radioactivity added to soil at the start of the present study. As part of the mass-balance studies, the ^{14}C-activity in the acetonitrile-extractable fraction as well as the tissue-residue (or nonextractable) fraction also were examined to follow the fate of [^{14}C]RDX absorbed by the earthworms. The ^{14}C-activity remaining in the tissue pellet was separated from the acetonitrile extract by centrifugation of the solvent–tissue suspension. The radioactivity in these fractions was analyzed using LSC; this value is expressed as disintegrations per minute (dpm). Radioactivity in the acetonitrile-extractable fraction of the earthworm was considered to represent RDX or its unbound degradation products, whereas radioactivity in the nonextractable fraction was considered to represent RDX degradation products that were bound to cellular constituents.

Analysis of ^{14}C-activity in soil and earthworm tissue samples

The ^{14}C-activity in soil or earthworm samples was determined using two methods. Wet combustion was performed using a glass and polytetrafluoroethylene apparatus containing hot acid, as described by others [22,37,38]. This setup consisted of a 100-ml, round-bottom flask with heating mantle, a gas inlet, a water-filled jacket condenser, and an outlet fitted with a separatory funnel used for liquid transfer. The outlet was connected to a KOH trap consisting of five test tubes (17 × 150 mm) attached in series. Each tube was filled with 10 ml of 0.5 M KOH containing a low concentration of thymolphtalein as a pH indicator (for changes in the alkaline range). Soil and earthworm samples were combusted in the following manner: Approximately 1 g of soil or 0.2 g of lyophilized ground earthworm tissue sample was taken. Then, 1.5 g of potassium dichromate ($K_2Cr_2O_7$) was added to each soil sample, and the samples were combusted in 25 ml of hot acid mixture (H_2SO_4:H_3PO_4 [3:2, v/v]) for 20 min. Earthworms were combusted in a 30-ml acid mixture. Four tubes, each containing 10 ml of KOH (0.5 M), were used to trap the evolved $^{14}CO_2$. A 1-ml sample from each of the KOH traps was analyzed using LSC. The detection limit of this method was 10×10^3 dpm/g tissue. The detection limit was improved to 1.3×10^3 dpm/g tissue later in the investigation, when the dry-combustion method became available to this laboratory. This technique involved use of the sample oxidizer that enabled analysis of smaller quantities of earthworm tissue (~0.02 g tissue) compared to the amount required for wet combustion (0.2 g tissue). Samples were prepared according to the manufacturer's instructions and counted using LSC.

BAF calculations

The BAF was determined using two approaches. The first approach involved use of the steady state–based distribution coefficient model, expressed as the ratio of the nonlabeled RDX concentration in the tissue (mg/kg) to the nonlabeled RDX concentration in the soil (mg/kg). The BAF is expressed as kg soil/kg tissue. The second approach was the BAF_K, or the net uptake of RDX, calculated as the ratio of the rate constant for the uptake of RDX (k_1) from soil by the earthworm to the rate constant for elimination of RDX (k_2) from the earthworm, assuming first-order, single-compartment exponential kinetics. The values for the RDX uptake rate constant (k_1), the elimination rate constant (k_2), and the BAF_K were calculated from model equations shown below. The goodness of fit of the models was determined from the coefficients of determination (r^2).

The tissue uptake rate constant (k_1) was derived from a single-compartment exponential uptake model for first-order kinetics as described by Bruns et al. [36]:

$$[RDX_T]_{total} = \frac{k_1}{k_2} \cdot [RDX_S] \cdot \left(1 - e^{-k_2 t}\right) \quad (1)$$

where $[RDX_T]_{total}$ is the total radioactivity (dpm/g dry wt tissue) in the earthworm tissue (^{14}C in extractable plus nonextractable fractions), k_1 is the uptake rate constant (g dry wt soil/g dry wt tissue/d), k_2 is the elimination rate constant (1/d), $[RDX_S]$ is the concentration of radioactivity in dry soil (dpm/g dry wt soil) at the end of RDX exposure (14 d), and t is the duration of uptake (d).

Either [^{14}C]RDX or its unbound metabolites were considered for the calculation of the elimination kinetics. These compounds were present in the acetonitrile-extractable fraction of the earthworm. Consequently, the elimination data of ^{14}C-radioactivity from earthworm extracts was used to estimate the tissue elimination rate constant (k_2) according to the following exponential elimination model:

$$[RDX_T]_{extract} = \left([RDX_T]_{SS} \cdot e^{-k_2 t}\right) + [RDX_T]_R \quad (2)$$

where $[RDX_T]_{extract}$ is the total radioactivity (dpm/g dry wt tissue) in the acetonitrile-extractable fraction of the earthworm, $[RDX_T]_{SS}$ is the extractable radioactivity in the earthworm (dpm/g dry wt tissue) under apparent steady-state conditions (defined as no significant change in tissue RDX concentrations with respect to period of exposure), and $[RDX_T]_R$ is the residual radioactivity (dpm/kg dry wt tissue) at the end of elimination phase.

Under steady-state conditions,

$$[RDX_T]_{SS} = \frac{k_1}{k_2} \cdot [RDX_S]_{SS} \quad (3)$$

$$BAF_K = \frac{k_1}{k_2} \quad (4)$$

where $[RDX_S]_{SS}$ is the radioactivity in the soil (dpm/g dry wt soil) at apparent steady-state conditions and BAF_K (g dry wt soil/g dry wt tissue) is the ratio of the tissue uptake rate constant (k_1) to the tissue elimination rate constant (k_2).

The biological half-life of RDX in the earthworm—that is, the time required for the organism to eliminate half the radioactivity absorbed under steady-state conditions ($t_{1/2}$, d)—was determined using the equation described by Lotufo and Lydy [28]:

$$t_{1/2} = \frac{\ln(0.50)}{k_2} \cong \frac{0.693}{k_2} \quad (5)$$

Nonlinear regression models were run in SYSTAT™ (Ver 7.01; SPSS) and KaleidaGraph™ (Ver 4.03; Synergy Software) for the iterative curve-fitting procedures. Histograms of the residuals and stem-and-leaf graphs were examined to ensure that normality assumptions were met. Untransformed data for tissue or soil RDX concentration were used in parameter calculations.

Statistical analysis

Analysis-of-variance procedures were used for the uptake and elimination data established over time and among concentrations for different exposure time series. Means separations were done using Fisher's least-significant-difference pairwise comparison tests. Statistical analyses were performed using SYSTAT (Ver 7.01). Student's t test was used for comparisons between treatments using Microsoft® Excel software. A significance level of $p \leq 0.05$ was accepted for all statistical analyses.

RESULTS AND DISCUSSION

Uptake of nonlabeled RDX in earthworms

Preliminary time-course studies were carried out to determine the length of time needed to achieve steady-state conditions for nonlabeled RDX uptake from SSL soil in which adult *E. andrei* were exposed to nominal RDX concentrations of 10, 100, 1000, and 10,000 mg/kg for varying periods of exposure (2–21 d). These studies showed that RDX concentrations in exposed earthworms achieved steady state on or after 7 d (data not shown). All earthworms survived the exposure and showed no signs of adverse effects, which was consistent with the results of earlier studies [4,6,7,22]. Consequently, adult *E. andrei* were exposed for 7 d to nominal RDX concentrations of 0, 1, 10, 100, 1,000, 3,000, and 10,000 mg/kg. The corresponding measured concentrations of RDX in SSL were 0, 0.66 ± 0.09, 10.6 ± 0.03, 102 ± 5, 967 ± 16, 2,850 ± 12, and 9,427 ± 103 mg/kg, respectively. Trace concentrations of MNX (<1% of RDX concentrations) were found in amended soil and earthworm samples from RDX-amended soil. Neither DNX nor TNX was detected in these samples. For ease of reference, the nominal concentrations are reported in the text and in the figures, unless otherwise stated. The concentration of RDX in tissue steadily increased with increasing soil RDX concentration; differences among several treatment groups were statistically significant ($p \leq 0.05$)

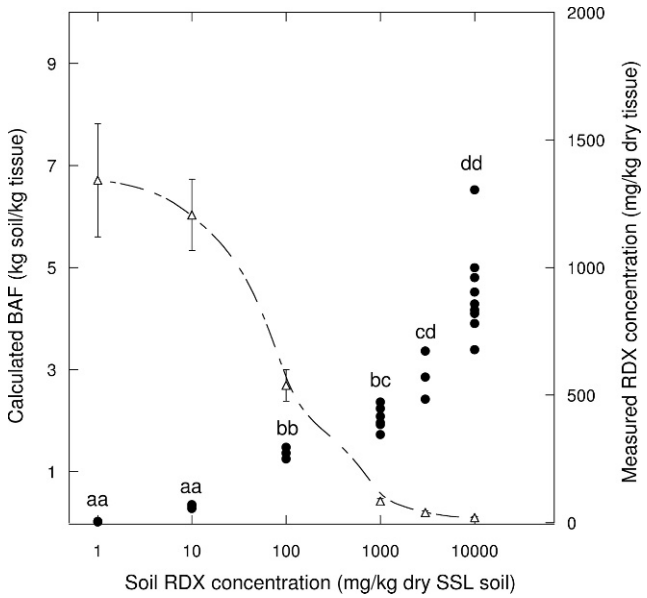

Fig. 2. Uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) in earthworms (*Eisenia andrei*) after the 7-d exposure to freshly amended soil. The RDX concentrations in tissue samples (●; right y axis) were determined using the U.S. Environmental Protection Agency Method 8330B [35] at different nominal RDX concentrations in a Sassafras sandy loam (SSL) soil (x axis). The calculated bioaccumulation factor (BAF) of RDX (△; left y axis) is expressed as the mean ± standard deviation (n = 3–8 replicates). The BAF of RDX in earthworms was 2.7 ± 0.3 at 100 mg RDX/kg soil. Common letters between treatment groups indicate no significant difference ($p > 0.05$) using analysis of variance and Fisher's least-significant-difference test.

(Fig. 2). The BAFs, expressed as distribution coefficients, decreased proportionally to the increase in the RDX concentration in soil and were 0, 6.7, 6.0, 2.7, 0.4, 0.2, and 0.1 for nominal soil RDX concentrations of 0, 1, 10, 100, 1,000, 3,000, and 10,000 mg/kg, respectively (Fig. 2). A similar relationship was reported by Sunahara et al. [22]. Based on the BAF distribution coefficients greater than one, the tissue accumulation of RDX occurred at measured RDX concentrations of 0.66, 10.6, and 102 mg/kg soil.

The results presented herein differ from those of Best et al. [24], who reported that the average BAF for RDX in the earthworm was equal to one, although closer examination of their data suggests that BAFs also may have changed as a function of RDX soil concentrations. The results of the latter study, together with the findings presented here, suggest that above a certain concentration of RDX in soil, small quantities of undissolved or crystalline RDX may be present in the exposure matrix and not accessible to the earthworms. This is consistent with the RDX tissue uptake data presented in Figure 2, showing that tissue concentrations of RDX increased 4.3-fold ($p \leq 0.05$) in earthworms exposed to soil RDX concentrations ranging from 10 to 100 mg/kg. In contrast, only a 1.4-fold increase ($p > 0.05$) was observed in tissue RDX concentrations when earthworms were exposed to soil RDX concentrations ranging from 100 to 1,000 mg/kg.

The HPLC analysis of tissue extracts indicated that the parent compound RDX as well as MNX, a reduced product of RDX degradation, were detected in earthworms exposed to different concentrations of RDX in soil (Fig. 3). Concentrations of MNX in earthworm tissues were directly proportional to the RDX concentrations in soil. A transient increase in MNX concentration was found in the tissue after a 1-d

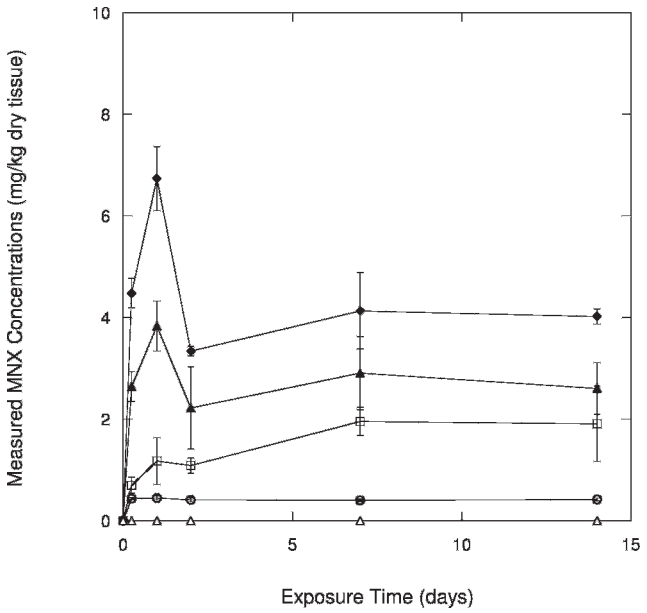

Fig. 3. Presence of hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX; a reduced product of hexahydro-1,3,5-trinitro-1,3,5-triazine [RDX]) in earthworms exposed to different concentrations of RDX in a Sassafras sandy loam soil for up to 14 d. The MNX concentrations in tissue following exposure to soil containing no RDX (△), 10 mg/kg (○), 100 mg/kg (□), 1,000 mg/kg (▲), or 10,000 mg/kg (♦) are shown. Data are expressed as the mean ± standard deviation (n = 3 replicates). If not visible, error bars are smaller than the symbol.

exposure to RDX, after which these concentrations decreased or attained a plateau between 7 and 14 d of the study. The peak of tissue MNX concentrations in the 1-d exposure was most evident in the 10,000 mg/kg treatment. These changes may represent the early phases of MNX equilibration in soils containing earthworms. It is doubtful that these effects are related to RDX degradation in the soil, because control studies indicated that no additional MNX was formed in RDX-amended soil incubated for up to 14 d without earthworms added.

Low levels of MNX and TNX were detected by Best et al. [24] in *E. fetida* exposed to field-collected soils containing a variety of energetic materials, including RDX. It is not clear from the latter study if the reported RDX metabolites were formed in the earthworm or originated in the energetic materials–contaminated soil and were taken up by the organism. In the present study, no TNX was detected in either soil or earthworm tissue using HPLC analyses. It should be noted, however, that after 14 d of exposure to RDX in soil, the tissue MNX concentrations represented less than 0.1% of the RDX concentrations in the earthworm. This amount was consistent with the quantity of MNX as a contaminant in the original RDX product (99.9% purity), and both compounds may have been coabsorbed by the earthworm from the amended soil.

Mass-balance studies using earthworms exposed to [^{14}C]RDX-amended soil

Time-series, mass-balance studies were conducted using the EAM containing *E. andrei* exposed to [^{14}C]RDX-amended SSL soil (100 mg/kg) for up to 14 d. The radioactivity was analyzed from samples of soil and earthworms and the CO_2 traps. Total radioactivity was calculated as the sum of the latter three fractions. Earthworm controls included soils amended with [^{14}C]RDX but with no earthworms added. The recovery of total radioactivity ranged from 90% ± 3% to 100% ± 6% compared to that added at the start of the experiment (Table 1). Most of the radioactivity (88–98%) remained in the soil, whereas from 1.7 to 3.4% was found in earthworms and from 0.1 to 1.8% as evolved $^{14}CO_2$. The recovery of radioactivity was not statistically different ($p > 0.05$) between soil samples with and without earthworms added (data not shown).

Table 1. Mass balance for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) as determined in the microcosm studies using the earthworm *Eisenia andrei* exposed to 100 mg [^{14}C]RDX/kg in a Sassafras sandy loam soil

Exposure duration	Recovery of ^{14}C-activity (%)			
	Soil[a]	Tissue[a]	CO_2 evolved[b]	Total recovery[c]
1 d	88 ± 3	1.7 ± 0.1	0.5	90 ± 3
2 d	98 ± 6	2.5 ± 0.5	0.1 (0; 0.2)	100 ± 6
7 d	90 ± 3	2.5 ± 0.0	0.9	94 ± 3
14 d	92 ± 6	3.4 ± 0.9	1.8 (0.9; 2.7)	97 ± 4

[a] Data are presented as mean percentages ± standard deviation (n = 3–6 replicates) based on the amount of [^{14}C]RDX added at the start of experiment.
[b] Cumulative ^{14}C-activity is the sum of the radioactivity measured in the internal and external CO_2 traps of KOH solution. Triplicate KOH samples were pooled because of the limited amount of radioactivity collected. Values in parenthesis indicate the individual means of two separate experiments.
[c] Total recovery is the sum of individual mass recoveries of [^{14}C] activity from soil, tissue, and evolved CO_2.

Uptake and elimination of [^{14}C]RDX in earthworms

The uptake of [^{14}C]RDX in earthworms was examined using *E. andrei* exposed to 100 mg [^{14}C-RDX]/kg SSL soil for up to 14 d. This RDX exposure concentration provided sufficient amounts of radioactivity for detection in the tissue samples. Figure 4 shows data from a representative study (experiment 3 in Table 2) and summarizes the temporal changes in radioactivity in the acetonitrile-extractable fraction as well as in the nonextractable fraction taken from [^{14}C]RDX-exposed earthworms. The radioactivity in the extracts (representing RDX and some of its metabolites) increased in a curvilinear fashion during the 14-d exposure to [^{14}C]RDX in SSL soil. Radioactivity in the nonextractable fraction was relatively low and increased to 42 × 10^3 dpm/g tissue by the end of the 14-d uptake phase of the experiment. Figure 4 shows the time course of total (sum of extractable and nonextractable fractions) uptake of [^{14}C]RDX in earthworms exposed to [^{14}C]RDX-amended soil. Analysis of the total uptake of [^{14}C]RDX in earthworms obtained from the three experiments revealed that the [^{14}C]RDX uptake rate constant (k_1) was 4.5 ± 2.4 g soil/g tissue/d (Table 2).

Results showed that the uptake of [^{14}C]RDX in earthworms can be described by a single-compartment kinetics model. The elimination of ^{14}C-activity from the earthworm was examined during days 14 to 28 of the experiment (i.e., when ^{14}C-containing earthworms were transferred to the nonamended SSL soil). Figure 4 summarizes the results and shows a rapid decrease of radioactivity in the extractable fraction of earthworms during the elimination phase. The radioactivity in the nonextractable fraction remained relatively stable (i.e., from 42 × 10^3 to 35 × 10^3 dpm/g tissue) during the 14-d elimination period. These results indicate that either the association of [^{14}C]RDX or its metabolites with the cellular

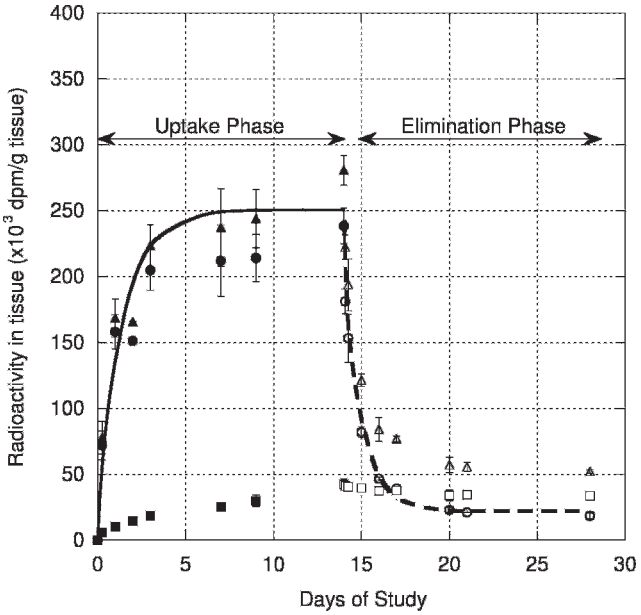

Fig. 4. Uptake and elimination of [^{14}C]hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) and its products in earthworm tissues. Shown are radioactivity (dpm) as measured from extracted tissue fractions (●, ○), radioactivity as measured from nonextractable fractions of earthworm tissues (■, □), and total radioactivity (acetonitrile-extractable + nonextractable fractions) from tissue samples (▲, △). Filled and unfilled symbols denote uptake and elimination of radioactivity, respectively. Solid line shows best-fit curve for total uptake of radioactivity (▲—▲); broken line shows best-fit curve for radioactivity in the extractable fraction (○– –○). Data are expressed as the mean ± standard deviation (n = 3 replicates). If not visible, error bars are smaller than the symbol.

constituents of the earthworm was irreversible or that the dissociation of these [^{14}C]RDX metabolites from this ^{14}C-complex was very slow. Further studies will be carried out to identify the [^{14}C]RDX metabolites in the nonextractable fraction. The persistence and toxicological relevance of these RDX metabolites in the earthworm also will require further investigation.

Analysis of the elimination of ^{14}C-activity from the earthworm indicated a rapid efflux (k_2 = 1.2 ± 0.5/d) of radioactivity from an average steady-state concentration (138 × 10^3 dpm/g) at the start of the elimination phase to a residual level (19 × 10^3 dpm/g) that remained in the earthworm until the end of the elimination phase (Table 2). Based on these data, 0.7 ± 0.4 d is required to eliminate half the [^{14}C]RDX absorbed by the earthworms.

The kinetically derived BAF_K of RDX was estimated using the ratio of paired uptake (k_1) to elimination (k_2) rate constants for each experiment. The BAF_K was 3.6 ± 0.5 (n = 3 experiments) and was similar to the BAF (2.7 ± 0.3) obtained when earthworms were exposed to 100 mg/kg nonlabeled RDX in SSL soil (Fig. 2). Recent preliminary studies (K. Savard et al., unpublished data) using earthworms exposed to 10 mg [^{14}C]RDX/kg SSL soil showed that the tissue uptake rate constant (k_1) was 9.1 g soil/g tissue/d and that the tissue elimination rate constant (k_2) was 1.2/d (asymptotic standard error = 0.1). The resulting BAF_K was 7.3 and was consistent with the BAF of 6.0 determined when earthworms were exposed to 10 mg/kg of nonlabeled RDX (Fig. 2). These results indicate that the BAF_K varies according to the soil RDX concentration (i.e., $[RDX_S]$). A similar conclusion was found based on calculation of the steady-state distribution coefficient BAF. Although the bioaccumulation potential or tissue uptake of RDX from soil was determined using two different approaches, they share a common exposure parameter—namely, the measured $[RDX_S]$. The $[RDX_S]$ was used to calculate the BAF (i.e., $[RDX_T]/[RDX_S]$), and the tissue RDX concentration $[RDX_T]_{total}$ parameter (shown in Eqn. 1) that also uses the steady-state RDX concentration in soil (i.e., $[RDX_S]_{SS}$). These soil RDX concentrations were based on acetonitrile extraction of soil samples. It is possible that the $[RDX_S]$ values reported here using the U.S. Environmental Protection Agency Method 8330B [35] may overestimate the actual RDX exposure concentration that is accessible to the earthworm in soil. Therefore, a more accurate exposure parameter, such as the interstitial water fraction of soil, should be considered. Preliminary ongoing studies suggest that this soil fraction becomes saturated with RDX (maximum solubility is 42 mg/L at 20°C) [39]) at soil total RDX concentrations greater than 40 to 50 mg /kg [40]. Whether or not the interstitial water fraction of soil plays a role in affecting RDX accumulation in earthworms, the RDX uptake data described in the present study indicates that earthworms can accumulate RDX from soil. Such accumulation can pose a risk for RDX exposure by higher-trophic-level receptors through the food-chain transfer.

CONCLUSION

The present study has demonstrated that the RDX BAFs, expressed as steady state–based distribution coefficients, ranged from 0.1 to 6.7, depending on the soil RDX concentration. These values were similar to those derived using the kinetic approach. Toxicokinetic studies using [^{14}C]RDX indicated a net accumulation of RDX by the

Table 2. Parameters of uptake and elimination for [^{14}C]hexahydro-1,3,5-trinitro-1,3,5-triazine ([^{14}C]RDX) in the earthworm *Eisenia andrei*[a]

	Body residue[b] (× 10^3 dpm/g tissue)		Rate constants[b]		Coefficient of determination (r^2)			
	$[RDX_T]_{SS}$	$[RDX_T]_R$	k_1 (g soil/g tissue/d)	k_2 (1/d)	Uptake	Elimination	BAF_K	$t_{1/2}$ (d)
Experiment 1	68 (6)	14 (4)	1.9 (0.1)	0.6 (0.1)	0.8	0.9	3.0	1.1
Experiment 2	153 (9)	17 (6)	6.6 (0.3)	1.6 (0.3)	0.8	0.9	4.1	0.4
Experiment 3	192 (7)	26 (4)	4.9 (0.2)	1.3 (0.2)	0.9	1.0	3.6	0.5
Mean ± SD	138 ± 63	19 ± 6	4.5 ± 2.4	1.2 ± 0.5			3.6 ± 0.5	0.7 ± 0.4

[a] $[RDX_T]_{SS}$ is the sum of the radioactivity in the acetonitrile-extractable fraction of the earthworm (dpm/g dry wt tissue) under apparent steady-state conditions, and $[RDX_T]_R$ is the residual radioactivity remaining in the extractable fraction of the earthworm (dpm/g dry wt tissue) at the end of the experiment. Rate constants were determined using the model specified in the text; k_1 is the total ^{14}C-activity uptake rate constant (g dry wt soil/g dry wt tissue/d) and k_2 the elimination rate constant (per day) for extractable ^{14}C-activity. BAF_K is the kinetics-based bioaccumulation factor, defined as k_1/k_2, and $t_{1/2}$ (d) is the eliminated half-life of the extractable ^{14}C-activity in the earthworms. SD is the standard deviation.

[b] Data are expressed as the mean ± standard deviation (n = 3) or with the asymptotic standard error indicated in parentheses.

earthworm *E. andrei*. Unidentified radioactive compounds were detected in the nonextractable fraction of earthworms exposed to [^{14}C]RDX in soil and indicated a tight or irreversible association between RDX metabolites and cellular constituents (proteins or nuclear material) in the organism.

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ROLE OF SOIL INTERSTITIAL WATER IN THE ACCUMULATION OF HEXAHYDRO-1,3,5-TRINITRO-1,3,5-TRIAZINE IN THE EARTHWORM *EISENIA ANDREI*

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Abstract—The uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) from soil by the earthworm *Eisenia andrei* was examined by using the equilibrium partitioning (EqP) theory and a three-compartment model including soil (S), interstitial water (IW), and earthworms (E). The RDX concentrations were measured using U.S. Environmental Protection Agency (U.S. EPA) Method 8330A and high-performance liquid chromatography (HPLC). The S-IW studies were conducted using four natural soils with contrasting physicochemical properties that were hypothesized to affect the bioavailability of RDX. Each soil was amended with nominal RDX concentrations ranging from 1 to 10,000 mg/kg. The HPLC analysis showed that the IW extracted from soil was saturated with RDX at 80 mg/kg or greater soil concentrations. The calculated S-IW coefficient (K_p) values for RDX ranged from 0.4 to 1.8 ml/g soil, depending on the soil type, and were influenced by the organic matter content. In the IW-E studies, earthworms were exposed to nonlethal RDX concentrations in aqueous media. The uptake of RDX by the earthworms correlated well ($r^2 = 0.99$) with the dissolved RDX concentrations. For the E-S studies, earthworms were exposed to RDX-amended soils used in the S-IW studies. The bioconcentration factors (BCF; ratios of E-to-IW RDX concentrations) were relatively constant (~5) up to 80 mg/kg soil RDX concentrations, which encompass the RDX saturation limit in the interstitial water of the tested soils. At this concentration range, the RDX uptake from interstitial water was likely dominated by passive diffusion and could be used as an indicator of bioavailability. Other mechanisms may be involved at greater RDX soil concentrations. Environ. Toxicol. Chem. 2010;29:998–1005. © 2009 SETAC

Keywords—Hexahydro-1,3,5-trinitro-1,3,5-triazine Bioaccumulation Bioavailability Bioconcentration Equilibrium partitioning

INTRODUCTION

Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) is a polynitramine explosive found as a contaminant at sites related to RDX manufacturing, use, and disposal. The RDX concentrations can range from very low levels to 3,500 mg/kg at some military firing and training sites and can reach up to 74,000 mg/kg at open burning/open detonation areas [1–6].

The toxicity of RDX to earthworms has been well documented [6–12]. Although RDX was not lethal to soil invertebrates such as adult enchytraeids up to approximately 20,000 mg/kg [13], adverse sublethal effects at lower concentrations have been reported. For example, exposure to RDX in soil can decrease juvenile production in *Eisenia andrei* [7,8], *Enchytraeus albidus* [14], and *Enchytraeus crypticus* [13], with the lowest-observed effect concentration values ranging from 15 to 3,715 mg/kg, depending on the test species.

Previous experiments demonstrated a limited accumulation potential for RDX in earthworms [1,15–17], as could be expected from its low log K_{OW} of 0.87 [18]. The biota-soil-accumulation factor (BSAF), typically expressed as the ratio of tissue to total soil concentrations [19], is often used to characterize the bioaccumulation potential of a chemical from soil to a soil-dwelling organism, such as the earthworm. Recent studies have shown, however, that the BSAF of RDX in earthworms decreased from 6.7 to 0.10 g soil/g tissue as the RDX concentration in soil increased from 1 to 10,000 mg/kg soil [1,15,16]. A varying BSAF value can increase the uncertainty of estimated food chain transfer potential for RDX during the ecological risk assessment at a contaminated site. Therefore, it is important to examine the approaches used to determine the BSAF value of RDX.

The U.S. Environmental Protection Agency (U.S. EPA) Method 8330A [20] (http://www.epa.gov/waste/hazard/testmethods/sw846/online/8_series.htm) is often used in ecotoxicity studies to estimate the RDX exposure concentration in soil [1,15,16]. This method, which is based on acetonitrile extraction, quantifies the total concentration of RDX that includes the nonsoluble (crystalline plus sorbed) and the water-soluble fractions of RDX. Therefore, U.S. EPA Method 8330A has the potential to overestimate the amount of RDX available to the exposed organism.

The equilibrium partitioning (EqP) theory has been used to assess the uptake of organic compounds by soil organisms [21,22]. This theory stipulates that the bioavailability of an organic compound having a log $K_{OW} < 5$ for uptake by a soil organism is determined by the fraction dissolved in the interstitial water [23]. Moreover, according to the EqP theory, dermal absorption of an organic chemical into the earthworm can be derived from the concentration in the interstitial water using the bioconcentration factor (BCF; Fig. 1) [24].

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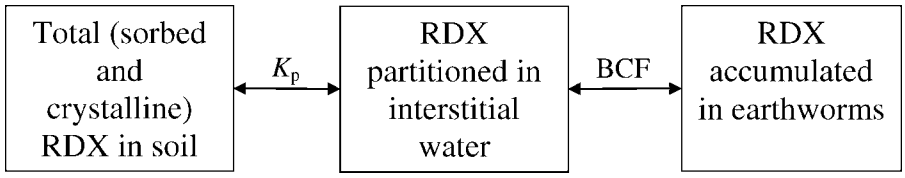

Fig. 1. Equilibrium partitioning (EqP) theory applied to hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX). K_p = soil-to-interstitial water partition coefficient; BCF = bioconcentration factor.

To improve our basic understanding of RDX bioaccumulation in soil, uptake of RDX in the earthworm was evaluated by considering independently the uptake of RDX in soil and from interstitial water and the sorption of RDX in soil (Fig. 1). The objectives of the present study were to quantify the RDX uptake in earthworms (*E. andrei*) using either the total RDX concentration in soil (BSAF) or the RDX fraction dissolved in interstitial water (BCF), using earthworm exposures in four natural soils with contrasting physicochemical properties, and to test the hypothesis that soil properties can affect the earthworm uptake of RDX such that the concentration of RDX in interstitial water can be used as an indicator of RDX bioavailability in soil.

MATERIALS AND METHODS

Chemicals and reagents

Hexahydro-1,3,5-trinitro-1,3,5-triazine (Chemical Abstracts Service [CAS] No. 121-82-4; 99.9% purity with <0.1% hexahydro-3,5-dinitro-1-nitroso-1,3,5-triazine; MNX) was supplied by the Defence Research and Development Canada (DRDC), Valcartier. The reference standards included RDX, MNX (CAS No. 5755-27-1), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX; CAS No. 80251-29-2), and hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX; CAS No. 13980-04-6) from AccuStandard. Reagent-grade calcium chloride was obtained from BDH™, and anhydrous ethyl alcohol was obtained from Commercial Alcohols Inc. High-performance liquid chromatography (HPLC)-grade acetone and acetonitrile were purchased from Caledon Laboratories. The American Society for Testing and Materials (ASTM) type I deionized water [25] was produced using a Super-Q™ water purification system (Millipore) or Zenopure Mega-90. Other reagents were obtained from commercial suppliers. Glassware was washed with phosphate-free detergent followed by rinses with acetone, nitric acid (10%, v/v), and ASTM type I water.

Preparation of RDX-amended aqueous samples

For the aqueous exposure studies, the RDX solutions were prepared using ASTM type I water. A saturated RDX solution (60 mg RDX in 1 L of water) was prepared and stirred overnight in darkness. This mixture was then passed through a 0.22-μm filter (Millipore), and the RDX concentration in the filtrate (stock solution) was confirmed by using HPLC, as described below. The stock solution of RDX was serially diluted to yield nominal concentrations of 1, 3, 5, 10, 20, 25, 30, 35, and 40 mg/L. The ASTM type I water was used as the carrier control.

Preparation of RDX-amended soil samples

Natural soils used in this study included Teller sandy loam (TSL; fine-loamy, mixed, active, thermic Udic Argiustoll soil, obtained from Payne County, Oklahoma, USA), Kirkland loam (KL; fine, mixed, superactive, thermic Udertic Paleustoll soil, also obtained from Payne County, Oklahoma), Webster clay loam (WCL; fine-loamy, mixed, superactive, mesic Typic Endoaquoll soil, obtained from Story County, Iowa, USA), and a sandy soil (DRDC; obtained from a Canadian military training facility in Val-Bélair, Quebec, Canada). Each soil was air dried, sieved on a 2-mm screen, and then stored at room temperature prior to use. Table 1 summarizes the key physicochemical characteristics of the soils used in the present study. The particle size distribution was determined by using the hydrometer method [26], and the organic matter (OM) content was estimated by weight loss following ignition [27]. The pH was measured using a 1:5 (v/v) suspension of soil in water [28].

The TSL, KL, WCL, and DRDC soils were individually amended with RDX using acetone as the carrier to attain nominal RDX concentrations ranging from 1 to 10,000 mg/kg. Individual solutions of RDX in acetone were poured evenly across the soil surface, ensuring that the volume of solution added did not exceed 15% (v/w) of the dry soil mass. The greatest concentration (10,000 mg/kg) was prepared in several steps using a stock solution of 40 g/L, each time not exceeding 15% (v/w) of soil weight. Acetone was allowed to volatilize for 2 h between the steps [13,16,29]. All treatment groups including the carrier control (no RDX added) received the same quantity of acetone. All amended soil batches were then kept in a

Table 1. Selected physicochemical characteristics of soils used in the present study

Soil identification[a]	Soil type	Sand (%) 0.08–2 mm	Silt (%) 0.002–0.08 mm	Clay (%) <0.002 mm	OM[b] (%)	WHC[c] (ml/100 g)	pH
TSL	Sandy loam	65	22	13	1.4	16	4.4
KL	Loam	38	42	19	1.5	29	5.7
WCL	Clay loam	33	39	28	5.3	38	5.9
DRDC	Sandy	94	5	1	1.2	23	5.5

[a] TSL = Teller sandy loam soil; KL = Kirkland loam soil; WCL = Webster clay loam soil; DRDC = sandy soil provided by Defence Research and Development Canada, Valcartier.
[b] Organic matter.
[c] Water-holding capacity.

darkened chemical hood for at least 48 h to allow acetone to volatilize [14]. Control treatment groups also included a negative control (no acetone added) for each experiment. Each soil batch was mixed using a three-dimensional rotary soil mixer for 18 h 1 day before the experiment. Nominal concentrations of RDX included 1, 5, 10, 25, 50, 75, 100, 1,000, and 10,000 mg/kg for the soil interstitial water studies; 1, 10, 100, 1,000, and 10,000 mg/kg for studies involving earthworms exposed to RDX in soil; and 5, 10, 25, and 50 mg/kg for earthworms exposed to RDX in interstitial water.

Soil interstitial water collection

Individual samples (200 g dry soil mass) of prepared TSL, KL, WCL, and DRDC soil batches were placed into separate Mason-type 500-ml glass jars, hydrated to 75% of the soil water-holding capacity (WHC), and hand mixed with a spatula. Each jar was covered by a lid perforated with approximately 10 holes (1 mm diameter each), and was kept in an environment-controlled incubator at $20 \pm 1^{\circ}C$ (SD) and 70 to 80% relative humidity, with a 16:8-h light:dark photoperiod cycle with a mean light intensity of 800 ± 400 lux. After 24 h of soil moisture equilibration, the RDX concentration in each treatment group was determined in triplicate using the acetonitrile extraction procedure described below. Triplicate soil samples were collected from each jar to extract the interstitial water according to the coupled filtration–centrifugation method described by Lock and Janssen [30]. Briefly, 10 g of each soil sample was placed into a separate Sera-Separa™ filter (10.8 cm long, 9 ml capacity; Evergreen Scientific). Each filter was inserted into a separate conical polypropylene tube for subsequent centrifugation using a Sorvall Super T21 (Sorvall, Mandel Scientific) set at 1,800 *g* for 45 min at 20°C. The filtrate was collected and passed through a 0.45-μm Millex™-HV cartridge (Millipore) to eliminate the precipitate. A fraction of the filtrate was then mixed with acetonitrile (1:1, v/v) before HPLC analysis.

Earthworm exposures to RDX in aqueous media

Earthworms, *E. andrei,* were obtained from Carolina Biological Supply™ and were cultured at room temperature in earthworm bedding (Magic Products) supplemented weekly with dry food (Magic Worm Food; Magic Products). Adult earthworms with developed clitellum and weighing between 454 and 581 mg (wet wt) were selected for the experiments.

Earthworms were exposed to a solution of ASTM type I water amended with RDX (40 mg/L) to evaluate the RDX uptake in earthworm tissues after different exposure periods. Five depurated earthworms per treatment group were exposed in each replicate ($n = 3$) glass Petri dish containing 5 ml of the RDX solution for 0.13, 0.25, 1, or 2 d. The test medium was not renewed during the exposure. A negative control (water only and earthworms) was included. Petri dishes were placed in darkness in an environment-controlled incubator at $20 \pm 1^{\circ}C$ and 70 to 80% relative humidity. The HPLC analysis of aqueous RDX solutions taken at the beginning and at the end of the exposure confirmed the stability of the RDX concentrations after 1 d. Earthworms were rinsed after each exposure period, blotted, weighed, frozen in a dry ice–ethanol bath, and then kept at $-80^{\circ}C$ until RDX extraction from the tissues and HPLC analysis. In a separate experiment, the procedure described above was applied using earthworms exposed for 1 d to different RDX concentrations ranging from 1 to 40 mg/L.

The uptake of RDX in earthworms was also examined using interstitial water samples prepared from the RDX-amended TSL soil treatments or carrier control batches. Samples of TSL soil from each treatment group were placed into separate plastic containers (1.2 kg soil per container) and then hydrated to 75% of the soil WHC. Each container was covered with a perforated lid and was kept for 24 h in a lighted, environment-controlled incubator (as described above) to attain a steady-state of soil hydration. After this period, the interstitial water was collected using the procedure described above (see *Soil interstitial water collection*). Approximately 15 soil samples from each soil concentration were centrifuged to obtain at least 15 ml interstitial water. Five depurated earthworms were placed in each replicate ($n = 3$) glass Petri dish containing 5 ml of the interstitial water sample representing the specific TSL soil treatment. The test medium was not renewed during the exposure period. After the 1-d exposure in darkness in an environment-controlled incubator (described above), all earthworms were rinsed, blotted, weighed, frozen in a dry ice–ethanol bath, and kept at $-80^{\circ}C$ until RDX extraction from the tissues and HPLC analysis. The RDX concentration in interstitial water was analyzed before and after the 1-d exposure.

Earthworm exposures to RDX-amended soils

Individual samples (60 g dry soil mass) of prepared TSL, KL, WCL, and DRDC soil batches were placed into separate glass jars, using the method described by Sarrazin et al. [16]. Three replicates were used for each treatment. Soils were hydrated to 75% of their WHC for 3 h before the addition of earthworms. Two grams of dry food was added to each test unit, and the soil was hand mixed with a spatula. Six earthworms acclimated in nonamended soils for 1 d before the exposure studies were placed into a separate glass jar containing the amended soil sample, and each jar was covered with a perforated lid. All jars were placed in an illuminated, environment-controlled incubator as described above. Earthworms were removed from the test jars after 0.25, 1, 2, 7, 14, 21, and 28 d of exposure and were depurated on a moistened filter paper for 1 d to ensure the absence of visible soil particles in the intestinal tract. The earthworms were then rinsed, blotted on filter paper, weighed, and frozen in a dry ice–ethanol bath. Also, soil aliquots (20 g) were collected from each test jar at the beginning and at the end of the experiment. Individual earthworms and soil samples were stored at $-80^{\circ}C$ and $-20^{\circ}C$, respectively, before chemical analyses.

Chemical analyses of RDX in soil, tissue, and aqueous media

Concentrations of RDX and its metabolites in soil were determined using the modified U.S. EPA Method 8330A [20] and as described elsewhere [3]. For quantifying RDX in the aqueous media, each RDX solution was mixed with acetonitrile (1:1; v/v) before HPLC analyses. The HPLC detection limits for RDX and its metabolites were 0.25 mg/kg dry soil and 50 μg/L aqueous media.

Concentrations of RDX and its metabolites in the earthworm tissue were determined using a modification of the method

described by Renoux et al. [31]. Briefly, earthworms were lyophilized, ground, rehydrated with distilled water, and sonicated in the dark after addition of acetonitrile. Samples were centrifuged (12,000 *g* for 10 min at 4°C), and 3.5 ml of the supernatant was mixed with 1.5 ml of a 16 g/L calcium chloride solution before filtration and analysis by HPLC. The limit of detection for RDX and its metabolites in the earthworms was 5 mg/kg dry tissue.

Parameter estimations and statistical analyses

The BSAF (expressed as g soil/g tissue), the soil-to-interstitial water partition coefficient (K_p, ml/g soil), and the BCF (ml/g tissue) were calculated using Equations 1 to 3, respectively

$$\text{BSAF} = \frac{[\text{RDX}_T]}{[\text{RDX}_S]} \quad (1)$$

$$K_p = \frac{[\text{RDX}_S]}{[\text{RDX}_{IW}]} \quad (2)$$

$$\text{BCF} = \frac{[\text{RDX}_T]}{[\text{RDX}_W]} \text{ or } \frac{[\text{RDX}_T]}{[\text{RDX}_{IW}]} \quad (3)$$

where $[\text{RDX}_T]$, $[\text{RDX}_S]$, $[\text{RDX}_{IW}]$, and $[\text{RDX}_W]$ are RDX concentrations in the tissue (expressed as μg/g tissue), the soil (mg/kg soil), the soil interstitial water (mg/L), and water (mg/L), respectively. The RDX concentrations in soil, interstitial water, or tissue measured at the end of exposure were used for all calculations.

Soil-property data were log-transformed to normalize distribution. The analysis of variance and Student's *t* test for pairwise means separation was used to detect significant differences among treatments. Pearson's analysis and uncorrected probabilities were used to identify significant correlations among the selected soil parameters (clay content, OM content, and pH) and the K_p values. The WHC was not included in these analyses because of its dependence on OM and clay content. A significance level of $p \leq 0.05$ was accepted for statistical tests. All statistical analyses were performed using measured chemical concentrations and SYSTAT™ 11.0 for Windows (SPSS) and JMP IN™ version 4.0 software (SAS).

RESULTS AND DISCUSSION

Partitioning of RDX in amended soils

Concentrations of RDX were determined in the interstitial water of the four natural soils amended with nominal RDX concentrations ranging from 1 to 10,000 mg/kg. Preliminary studies showed that the RDX concentration in the soil interstitial water increased over time and reached a plateau after 1 d of soil hydration (data not shown). The interstitial water samples from TSL, KL, WCL, or DRDC soils were therefore collected at that time using the coupled filtration–centrifugation method. Concentrations of RDX in the soil interstitial water samples increased with increasing RDX concentrations in the amended soils (Fig. 2), approximately up to the aqueous solubility limit of RDX (42 mg/L at 20°C) [18]. Based on the data shown in Figure 2, the K_p values for RDX in each soil type were calculated as the ratio of the RDX concentration in a given soil sample to the concentration of RDX in the nonsaturated interstitial water sample (Eqn. 2). For this analysis, only data ranging from 0 to the limit of RDX saturation in each soil (20 to 80 mg/kg, depending on the soil type) were used. Statistical analyses revealed that the K_p values were significantly different (Student's *t* test; $p \leq 0.05$) among the four soils tested. The K_p value (ml/g) for RDX was greatest in WCL (1.8), followed by KL (0.9), TSL (0.6), and DRDC (0.4) soils, and indicated that the WCL soil had the lowest bioavailability for RDX compared with the other soils tested.

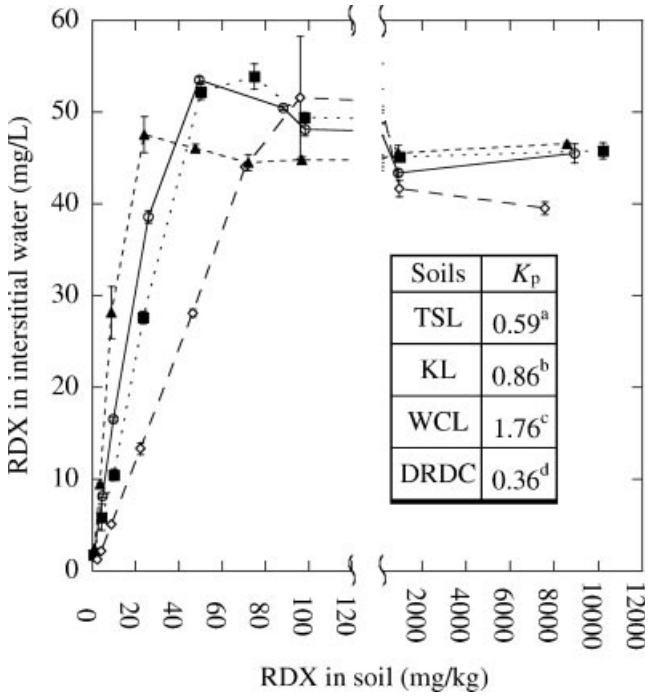

Fig. 2. Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) partitioned to soil interstitial water at different soil RDX concentrations (mg/kg) and soil types (circles, Teller sandy loam [TSL]; squares, Kirkland loam [KL]; lozenges, Webster clay loam [WCL]; triangles, sandy soil [DRDC]) after 1 d of soil hydration. The K_p (soil-to-interstitial water partition coefficient, ml/g) is the concentration of RDX in soil divided by the concentration of RDX in interstitial water. Different letters show significant differences between soil types (Student's *t* test, $p \leq 0.05$). Data are expressed as mean ± standard deviation ($n = 3$ replicates). If not visible, error bars are smaller than the symbol.

The OM, clay, and soil pH are known to play important roles in the sorption of organic compounds in soils [32,33]. Correlation analyses revealed that the K_p value was correlated strongly and significantly ($r = 0.978$; $p = 0.022$) with the OM content. The effect of clay content on RDX sorption was strong but not statistically significant ($r = 0.729$, $p = 0.271$; Table 2). This contrasted with earlier reports that suggested

Table 2. Pearson correlations and corresponding uncorrected probabilities for hexahydro-1,3,5-trinitro-1,3,5-triazine-amended soil-to-water partition coefficients (K_p) with clay, organic matter content, or pH of the four natural test soils

Soil properties	Correlation coefficients (*r*)	Uncorrected probability (*p*)
Clay	0.729	0.271
Organic matter	0.978	0.022
pH	0.546	0.454

a predominating role of clays in the sorption of RDX in soil [33–36]. The relationship between soil pH and the K_p value was weak and nonsignificant ($r = 0.546$, $p = 0.454$). Based on a limited data set, these findings indicated that the bioavailability of RDX in the soil interstitial water was influenced by the RDX sorption to the OM in the four soils tested.

Uptake of RDX by earthworms exposed in aqueous media

The BCF values for RDX were determined using RDX-amended water samples and interstitial water extracted from RDX-amended soil. A time course study was done using destructive sampling of earthworms exposed to RDX dissolved in water at concentrations approaching maximal aqueous solubility (40 mg/L) for up to 2 d. Concentrations of RDX in water attained an apparent steady state after a 1-d exposure (Fig. 3A). Therefore, this exposure period was chosen to determine RDX uptake by the earthworms in interstitial water that was extracted from TSL soil amended with different concentrations of RDX. The initial measured RDX concentrations in the interstitial water samples were 9.1 ± 0.01, 18 ± 0.1, 53 ± 0.1, and 50 ± 0.2 mg/L, corresponding to nominal soil RDX concentrations of 5, 10, 25, and 50 mg/kg, respectively. The corresponding RDX concentrations at the end of the study were 4.7 ± 0.23, 8.5 ± 0.22, 26 ± 1.3, and 24 ± 0.3 mg/L, respectively. In a separate study, earthworms were exposed for 1 d to different RDX concentrations in amended deionized water. Concentrations of RDX in tissue, in interstitial water, and in deionized water at the end of the exposure period are shown in Figure 3B. These data showed that uptake of RDX by the earthworms correlated strongly with the dissolved RDX concentrations in the interstitial water or the ASTM type I water ($r = 0.96$, $p = 0.0001$).

The calculated BCF (Eqn. 3) for the earthworms exposed separately in interstitial water from RDX-amended soil and in amended water was 13 ± 1 ml/g dry tissue or 2 ± 0.1 ml/g wet biomass. These results are similar to the BCF values for RDX (ml/g wet tissue) of 2.1 and 2.4 established in studies with channel catfish (*Ictalurus punctatus*) and aquatic oligochaetes (*Lumbriculus variegatus*), respectively [37].

Uptake of RDX by the earthworms exposed in soil

The RDX accumulation in earthworms from each soil type was evaluated on the basis of the BSAF determined under steady-state conditions (Eqn. 1). To determine the duration necessary to achieve steady-state conditions, earthworms were exposed to RDX in soils for up to 28 d. The uptake of RDX by the earthworms approached a steady state (indicated by the leveling off in tissue RDX concentrations) between 2 and 7 d from the start of exposure in WCL soil amended with RDX concentrations of 10, 100, or 1,000 mg/kg (Fig. 4A). Similar results were obtained in studies with the other three soils (data not shown). Therefore, a 7-d exposure period was chosen for quantifying the RDX bioaccumulation in all soils tested. For each soil, the RDX uptake in tissue increased from nondetectable concentrations to 1,514 mg/kg dry tissue, as the nominal RDX concentrations in soil increased from 1 to 10,000 mg/kg dry soil (Fig. 4B). The HPLC analyses showed that neither DNX nor TNX was found in the soil or the earthworm tissue samples; however, MNX was detected to a maximum concentration of 11 mg/kg when the earthworms were exposed to soil RDX concentration of 10,000 mg/kg. Similar results have been obtained by other authors [1,16]. Control studies showed that there was no MNX formed in RDX-amended soil incubated for up to 14 d without earthworms. However, MNX was present as a contaminant in the original RDX product (99.9% purity), so both compounds could be taken up by the earthworms from the amended soils.

The BSAF values for RDX were calculated for each treatment group (Table 3). If the bioavailable and total (acetonitrile-extractable) soil RDX concentrations were directly related, then increases in soil RDX concentrations would be associated with increases in tissue RDX concentrations and thus would result in a constant BSAF (according to Eqn. 1). The results presented here showed that the BSAF values were not constant and decreased from 13 to 0.05 g soil/g tissue as the RDX concentrations in soil increased from 1 to 10,000 mg/kg. These results are consistent with findings of earlier studies [1,16] and indicate that the total soil RDX concentration, as measured using acetonitrile extraction, does not specifically represent the

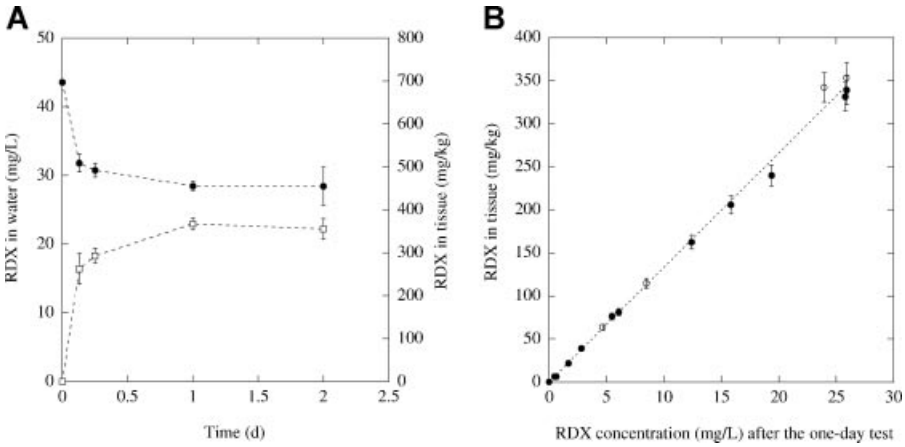

Fig. 3. Uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by earthworms exposed in aqueous media. (**A**) A time-series change in RDX concentrations in water (circles, left *y*-axis mg/L) and the earthworms (squares, right *y*-axis; mg/kg) exposed to single RDX concentration of 40 mg/L. (**B**) Linear relationship showing RDX uptake = 13.3 [RDX concentration in aqueous media] − 0.1 ($r^2 = 0.99$) by earthworms after a 1-d exposure in American Society for Testing and Materials type I water (solid circles) or in interstitial water extracted from RDX-amended soil (open circles). Data are expressed as mean ± standard deviation ($n = 3$ replicates). If not visible, error bars are smaller than the symbol.

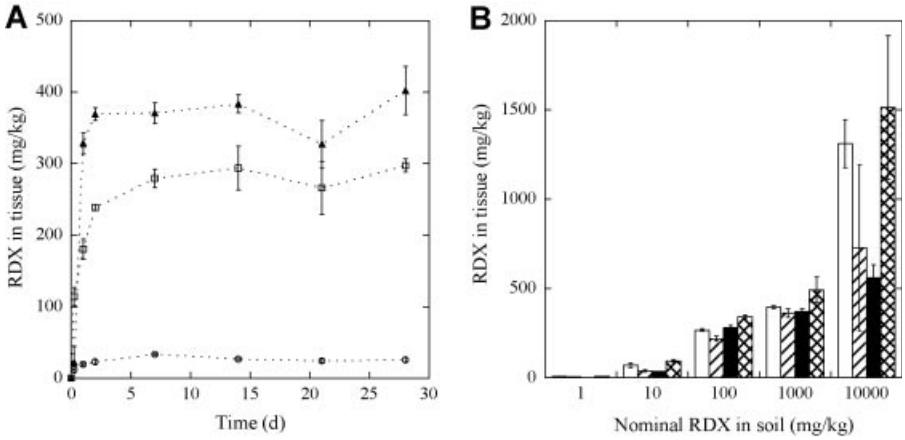

Fig. 4. Uptake of hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) by earthworms exposed in soil. (**A**) A time-series RDX uptake by earthworms (expressed as mg/kg) exposed to RDX concentrations 10 (circles), 100 (squares), and 1,000 (triangles) mg/kg in Webster clay loam soil. (**B**) RDX uptake (expressed as mg/kg) by earthworms exposed in natural soils (open columns, Teller sandy loam; diagonally hatched columns, Kirkland loam; solid columns, Webster clay loam; cross-hatched columns, sandy soil for 7 d. Data are expressed as mean ± standard deviation ($n = 3$ replicates).

bioavailable form of RDX. It is conceivable that the total soil RDX concentration represents two fractions of RDX in soil, a bioavailable fraction and another that is nonaccessible (e.g., undissolved or adsorbed RDX). In such a case, the increase in total RDX concentrations in soil relative to the constant concentrations of RDX in the tissue would lead to decreases in the BSAF values, as calculated with Equation 1.

Variations in the BSAF values were soil specific and decreased in the order DRDC > TSL > KL > WCL at RDX concentrations of 10 mg/kg (Table 3). The smallest BSAF was determined for WCL soil, which had the greatest OM content and the lowest bioavailability of RDX among soils tested in these studies. A similar inverse relationship between BSAF and OM was found in other studies [38–42]. At 100 mg/kg or greater RDX concentrations in soil, the trend was less clear (Table 3).

Concentrations of RDX dissolved in interstitial water of amended TSL, KL, WCL, and DRDC soils (shown in Fig. 2) were used to determine the RDX uptake in the earthworms exposed to RDX in soil. Interstitial water RDX concentrations correlated strongly and significantly with tissue RDX concentrations in nominal soil treatments of 1 and 10 mg/kg ($n = 15$, $r = 0.96$, $p = 0.0001$). In contrast, there were no significant correlations between RDX concentrations in interstitial water and in earthworms for nominal soil treatments of 100, 1000, and 10,000 mg/kg. These results indicate that the RDX partitioning in the soil interstitial water plays a determining role in RDX uptake by the earthworms from soil, up to the limit of RDX saturation in the interstitial water (i.e., below 100 mg/kg).

The BCFs were determined for each soil type and for each soil exposure concentration (Table 4). For nominal soil RDX concentrations of 1 and 10 mg/kg, the statistical analyses showed no significant differences in BCFs among soils tested ($n = 21$, analysis of variance and Student's t test, $p > 0.05$). The BCF values for all tested soils increased with increasing nominal RDX concentrations of 100, 1,000, and 10,000 mg/kg. Differences among many of those treatments were statistically significant (Table 4). These data and those shown in Figure 2 indicate that passive diffusion across the earthworm integument may be the predominant mechanism of RDX uptake from interstitial water at 80 mg/kg or lower soil RDX concentrations. The possible contribution of other xenobiotic uptake mechanisms (e.g., absorption and diffusion in the gut following soil ingestion) [23,24,43] could contribute at higher soil RDX concentrations. Further studies would be required to elucidate these mechanisms.

Table 3. Biota-soil-accumulation factor (BSAF) values for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) determined in studies with the earthworm *Eisenia andrei* exposed in four natural soils

	Nominal RDX concentrations in soil (mg/kg)				
Soil types[a]	1	10	100	1,000	10,000
TSL	9.0 ± 1.0A,W	8.0 ± 1.0A,W	2.8 ± 0.1A,X	0.43 ± 0.03A,Y	0.15 ± 0.02A,Y
KL	4.7 ± 0.3B,W	5.0 ± 0.7B,W	2.4 ± 0.2B,X	0.39 ± 0.03A,Y	0.08 ± 0.05AB,Y
WCL	ND[b]	3.2 ± 0.1B,W	3.1 ± 0.1A,X	0.39 ± 0.03A,Y	0.05 ± 0.01B,Z
DRDC	13 ± 1C,W	15 ± 2C, X	3.9 ± 0.3C,Y	0.58 ± 0.09B,Z	0.19 ± 0.07A,Z

BSAF (g soil/g tissue) is the RDX concentration in tissue (μg/g tissue) divided by the RDX concentration measured in soil (mg/kg soil). Data are expressed as the mean ± standard deviation ($n = 3$). Different capital letters (A, B, C) indicate significant differences between soil types having a same soil RDX concentration (analysis of variance [ANOVA] followed by a Student's t test, $p \leq 0.05$). Different capital letters (W, X, Y, Z) indicate significant differences between different soil RDX concentrations within the same soil type (ANOVA followed by a Student's t test, $p \leq 0.05$).

[a] TSL = Teller sandy loam soil; KL = Kirkland loam soil; WCL = Webster clay loam soil; DRDC = sandy soil provided by Defence Research and Development Canada, Valcartier.

[b] RDX was not detected (ND) in earthworm tissue. Limit of detection in tissue = 5 μg/g dry tissue.

Table 4. Bioconcentration factor (BCF) values for hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) determined in studies with the earthworm *Eisenia andrei* exposed in four natural soils

	Nominal RDX concentrations in soil (mg/kg)				
Soil types[a]	1	10	100	1,000	10,000
TSL	5.5 ± 0.1A,X	5.0 ± 0.9A,X	5.5 ± 0.1A,X	9.1 ± 0.2A,Y	29 ± 3AB,Z
KL	4.0 ± 0.1A,X	4.3 ± 0.6A,X	4.4 ± 0.3B,X	8.0 ± 0.5A,XY	16 ± 10BC,Y
WCL	ND[b]	5.6 ± 0.1A,X	5.4 ± 0.3A,X	8.9 ± 0.3A,Y	14 ± 2C,Z
DRDC	4.6 ± 0.2A,X	5.5 ± 0.5A,X	7.6 ± 0.2C,X	11 ± 2B,X	33 ± 9A,Y

BCF (ml/g tissue) is the RDX concentration in tissue (μg/g tissue) divided by the RDX concentration in interstitial water (mg/L). Data are expressed as the mean ± standard deviation ($n = 3$). Different capital letters (A, B, C) indicate significant differences between soil types having a same soil RDX concentration (ANOVA followed by a Student's t test, $p \leq 0.05$). Different capital letters (X, Y, Z) indicate significant differences between different soil RDX concentrations within the same soil type (ANOVA followed by a Student's t test, $p \leq 0.05$).

[a] TSL = Teller sandy loam soil; KL = Kirkland loam soil; WCL = Webster clay loam soil; DRDC = sandy soil provided by Defence Research and Development Canada, Valcartier.

[b] RDX was not detected (ND) in earthworm tissue. Limit of detection in tissue = 5 μg/g dry tissue.

CONCLUSIONS

The uptake of RDX in earthworms was evaluated by considering independently the RDX partitioning in three compartments, including soil—interstitial water, soil—earthworm, and interstitial water—earthworm. The RDX partitioning coefficients (K_p) determined in four natural soils with contrasting physicochemical properties and various RDX concentrations correlated strongly and significantly with the soil OM content. The results showed that the RDX concentration in the interstitial water played a determining role in RDX uptake by the earthworms exposed to 80 mg/kg or lower soil RDX concentrations, which is consistent with EqP theory. At this concentration range, the RDX uptake from interstitial water was likely dominated by passive diffusion and could be used as an indicator of bioavailability. Other mechanisms may be involved at greater RDX soil concentrations.

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